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The effect of UV-B on freezing tolerance in Arabidopsis thaliana

The effect of UV-B on freezing tolerance in *Arabidopsis thaliana*

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Abstract

Cold stress causes significant crop losses each year. Some plants can withstand this stress through the upregulation of genes which result in cold acclimation. This process involves the C-REPEAT BINDING FACTOR (CBF) regulon and *COLD RESPONSE* (*COR*) gene expression which protect the plant from low temperature damage. The signalling pathway that leads to cold acclimation is therefore of interest when investigating how to protect plants from low temperature stress. When multiple signalling pathways converge in a process known as crosstalk, enhanced resistance to a stress can result. Previous studies have shown that UV-B can enhance the survivability of *Rhododendron* at low temperatures and increase expression of protective enzymes involved in cold resistance in *Triticum aestivum*. This phenomenon has not been investigated in the model species, *Arabidopsis thaliana*. Here, the interaction of UV-B and cold acclimation in Arabidopsis is explored. An enhancement of *COR15a* and *COR47* transcript abundance was observed when cold acclimation occurred in the presence of UV-B, but this was not accompanied by increased plant survival following freezing stress at -6 ^{o}C. The role of flavonoids in cold and UV-B signalling was also analysed as flavonoids are known to be involved in both plant freezing tolerance and UV-B protection. Mutants deficient in flavonoid biosynthesis, *transparent testa* 4 (*tt4*) and *tt7* showed decreased survival following a freezing stress. This response was further exacerbated by UV-B treatment. The combination of cold and UV-B resulted in greater levels of *CHALCONE SYNTHASE* (*CHS*) transcript and flavonoid accumulation than either treatment alone, suggesting a synergistic interaction. Together, these data suggest that crosstalk exists between low temperature and UV-B signalling and that UV-B could be used conditionally to enhance cold acclimation and increase freezing tolerance in Arabidopsis.

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Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED:  DATE: 04/07/2019

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List of abbreviations

˙OH	- Hydroxyl radical
AP2/ERF	- APETALA2/ETHYLENE-RESPONSE ETHYLENE-BINDING FACTOR
bHLH	- Beta-helix-loop-helix
CA	- Cold acclimated
CAMTA	- CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR
CBF	- C-REPEAT BINDING FACTOR
CBLs-CIPKs	- CALCINEURIN B-LIKE CIPKs
CCA1	- CIRCADIAN CLOCK ASSOCIATED 1
CHI	- CHALCONE ISOMERASE
CHS	- CHALCONE SYNTHASE
COLD1	- CHILLING-TOLERANCE DIVERGENCE 1
COP1	- CONSTITUTIVELY PHOTOMORPHOGENIC 1
COR	- COLD RESPONSE
CPD	- Cyclobutene pyrimidine dimers
CRLK 1/2	- CALCIUM/CALMODULIN-REGULATED RECEPTOR-LIKE KINASE
CRLK1/2	- CALCIUM/CALMODULIN- REGULATED RECEPTOR – LIKE KINASE
CRPK1	- COLD RESPONSIVE PROTEIN KINASE
Cry	- Cryptochromes
DRE	- DEHYDRATION RESPONSE ELEMENT
DREB	- DEHYDRATION RESPONSE ELEMENT BINDING PROTEIN
F3’H	- FLAVONOID 3-HYDROXYLASE
FHY3	- FAR-RED ELONGATED HYPOCOTYL 3
FLS	- FLAVONOL SYNTHASE
GA	- Gibberellic acid
GA2ox	- GIBBERELLIC ACID 2 OXIDASE

H^2O^2 - Hydrogen peroxide

HOS1 - HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE

HY5 - ELONGATED HYPOCOTYL 5

HYH - HY5 HOMOLOG

ICE1 - INDUCER OF CBF EXPRESSION 1

INAS - Ice nucleation active substance

LEA II - LATE EMBRYOGENESIS ABUNDANT II

LHY - LATE ELONGATED HYPOCOTYL

LOR - Loss of osmotic responsiveness

LTRE - Low temperature responsive element

MAPK - MITOGEN ACTIVATED PROTEIN KINASE

MEKK1 - MITOGEN ACTIVATED PROTEIN KINASE KINASE KINASE 1

MKK2 - MITOGEN ACTIVATED PROTEIN KINASE KINASE 2

MPK4 - MITOGEN ACTIVATED PROTEIN KINASE 4

Phot - Phototropins

Phy - Phytochromes

PIF - PHYTOCHROME INTERACTING FACTOR

qPCR - Quantitative Polymerase Chain Reaction

RGA1 - RICE G-PROTEIN ALPHA SUBUNIT

ROS - Reactive oxygen species

SPA 1-4 - SUPPRESSOR OF PHYTOCHROME A-105

TLC - Thin layer chromatography

TT4 - TRANSPARENT TESTA 4

TT7 - TRANSPARENT TESTA 7

UV-B - ULTRAVIOLET B

UVR8 - UV RESISTANCE LOCUS 8

WT	- Wild type
ZAT12	- ZINC FINGER OF ARABIDOPSIS THALIANA 12
ZTL	- ZEITLUPE

CHAPTER 1: INTRODUCTION

The seasonal temperature variation experienced by plants presents a myriad of challenges which has resulted in the evolution of adaptations to closely monitor seasonal variation. This monitoring allows plants to adjust their phenology to help them survive through changing climates. During winter months, a prominent abiotic threat to plant survival is the cold, through chilling and freeze-damage (Korn *et al*., 2008). This results in reduced crop yield and is one of the many contributing factors threatening global food security. Other factors include heat and drought stress due to global warming. This is predicted to increase the average temperature over the next century (Table 1) and have a negative impact on crop yield (Figure 1). The United Nations population growth estimates predict a population increase of 9.7 billion by 2050 and 11.2 billion by 2100 (Figure 2) (IPCC, 2007; United nations, 2017). When crop yield losses from global warming projections are taken in confluence with world population estimates over the coming century then the importance of food security becomes a problem of paramount importance.

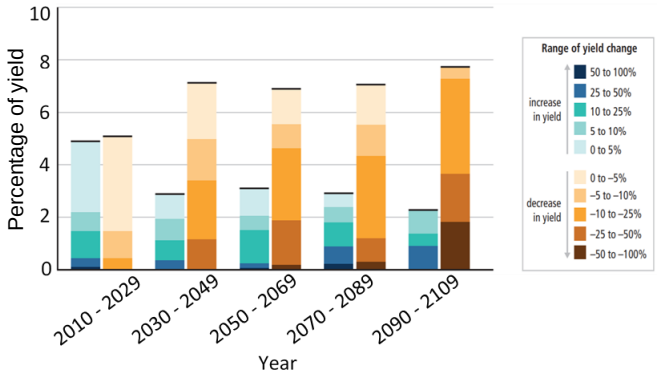

Figure 1. Projected crop yield increase and decrease over time due to climate change. Data is based on changes in crop yield from the 20th century. Adapted from IPCC (2014).

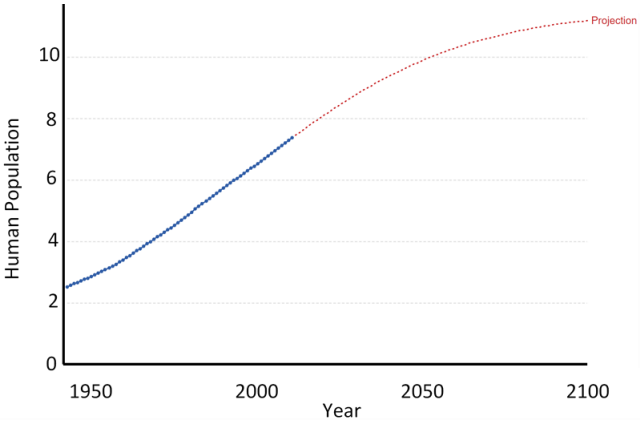

Figure 2. The revised 2017 projected human population increase based on data collected between 1950 – 2015. Blue dotted line indicates known population size, red dotted line indicates predicted population increase. Adapted from United Nations world population prospects, 2017 revision.

Table 1. Three IPCC predicted temperature increase scenarios for 2090 - 2099 based on information collected 1980 – 1999 (IPCC, 2007).

IPCC predicted scenario	Best estimate Temperature increase (°C)	Likely range temperature increase (°C)
Low	0.6	0.3 – 0.9
Medium	2.4	1.4 – 3.8
High	3.4	2.0 – 5.4

As climate change continues to contribute to increases in temperatures around the world perhaps counter intuitively, sudden frosts are becoming more prominent which are furthering agricultural losses each year (Warmund *et al.*, 2008). A precocious spring phenology means plants are germinating earlier in the season (Warmund *et al*., 2008) thus exposing plants to a larger range of temperatures than would be expected later in the season. This was demonstrated by the 2007 Eastern spring freeze in the US (Gu *et al.*, 2008). Immature tissues are especially susceptible to cold damage and thus the eastern spring freeze was responsible for widespread agricultural damage across America. Agricultural losses in North Carolina alone were approximately $111.7 million. During this time, it was noted that the spring freeze was characterized by seasonally high temperatures followed in quick succession by low temperatures Gu *et al*. (2008). Other large frosts such as the Swiss Rhone valley frost in 2012 concur with the paradigm of exceptionally warm temperatures early in the season followed by a sudden drop in temperature causing plant losses (Meier *et al*., 2018).

Solutions to thwart agricultural losses from abiotic stresses such as drought and increased temperature include increasing the geographical range in which crops are grown. Many plant species have already been demonstrated to be moving poleward or increasing altitude putatively due to increasing global temperatures (Walther *et al*., 2002). Moving crops poleward in hope to reduce the exposure to drought and increased temperatures is a possibility to reduce yield losses but this brings other inherent risks. Cultivars from the tropics have in general a less developed cold response compared to cultivars from temperate and poleward regions and thus the risk of chilling and freezing damage increases the further poleward they are moved.

One potential solution to reduce agricultural losses may be to increase UV-B supplementation to plants. Many greenhouse grown plants have reduced UV-B exposure due to greenhouse materials which attenuate UV-B light. Interesting studies exist which advocate the use of UV-B to mitigate freezing damage. Wargent *et al*., (2011) has shown that UV-B treatment of seedlings increased harvestable yield and photosynthetic output in field-grown *Lactuca sativa* (lettuce). These data suggest that UV-B treatment prior to planting may enhance the robustness of field-grown crops. Dunning *et al*. (1994) demonstrated that Rhododendron increased survival during freezing temperatures when treated with supplementary UV-B prior to exposure of freezing temperatures. Wheat has also been shown to respond to UV-B increasing its freezing tolerance by enhancing reactive oxygen species (ROS) scavenging enzymes Yang *et al*., (2007). In order to safeguard agriculture against crop losses from chilling and freezing temperatures, a fundamental understanding of the signalling pathways involved in plant protection is required.

Understanding this signalling network would enable the elucidation of new genetic targets which could be exploited through plant breeding or genetic modification to protect plants against freezing temperatures. This could be achieved by enhancing the plant's natural defences against cold stress through over expression of key genes, or through the implementation of new initiatives in crop management which exploit naturally occurring defence mechanisms.

1.1 Low temperatures

Cold-sensitive plants are broadly split into two categories based on their ability to cope with cold- chilling sensitive and freezing sensitive. Chilling stress refers to plants that become injured at temperatures below 10 °C but above 0 °C. Plants that suffer chilling injury are normally endemic to the tropics. Among the injuries that can be expected from chilling stress are wilting, chlorosis, sterility and occasionally death (Levitt, 1980; Knight and Knight, 2012). The primary site of injury is the chloroplast in which the organelle swells and disorganisation occurs (Kimball and Salisbury, 1973). Freezing injury occurs at temperatures below 0 °C and involves cellular dehydration and membrane injury. As the temperature falls below 0 °C, extracellular water is prone to freezing as it has a lower solute concentration than the intracellular water. The higher solute concentration depresses the freezing point inside the cell. Biological ice nucleation which causes freezing in plants is caused predominantly by an ice nucleation active substance (INAS) and this process is known as heterogenous ice nucleation. The presence of an INAS can be external or internal. External freezing occurs when moisture is present on the leaf surface. When moisture is in contact with an INAS and at an appropriately low enough temperature, ice nucleation can be initiated. As the leaf cuticle is a natural ice barrier, for the ice to spread from the external surface of the plant to the extracellular fluid it must enter through openings in the leaf. Openings occur when the leaf cuticle becomes damaged or a stomatal pore or hydathode provides access to extracellular fluid (Pearce and Fuller, 2001). Internal ice nucleation from INAS has been suggested to be less common but still possible due to bacteria. Some bacterial membranes contain anchored proteins that act as an INAS (also referred to as INA-bacteria) and this may allow ice formation to occur without an external ice influence (Lindow *et al*., 1989; Hirano and Upper, 2000). Without the presence of INAS, ice nucleation will not occur until approximately -40 °C (also known as homogenous ice nucleation) (Wisniewski *et al*., 2014).

1.2 Freezing Injury

Once the extracellular spaces become frozen, there is a reduction in water potential (Ψ) outside the cell. Ψ reduces because the chemical potential of ice is lower than that of water (Hansen and Beck, 1988). Therefore, osmotically active water exits the cell to address the Ψ disparity between the inside and outside of the cell. The movement of water molecules increases the concentration of solutes inside the cell depressing the freezing point by 1.86 °C per mole of solute dissolved per kg of water (Chang, 1999). The lower the temperature falls below 0 °C, the more osmotically active water is removed from the cell until at -10 °C, 90% of the osmotically active water is removed (Thomashow, 1999). The water relocated from inside the cell to the extracellular space then freezes.

The movement of water to the cell's extracellular space creates a multitude of problems. Initially, chemical stress occurs due to the initial movement of water outside the cell and culminates in cellular dehydration. As membranes are composed of bipolar phospholipids which rely on a hydrophobic and hydrophilic interactions to maintain a bilayer, dehydration can disrupt the biological membrane. Dehydration from extracellular freezing can cause lamellar to hexagonal II phase transition disrupting cellular communication and impair membrane integrity (Figure 3) (Gordon-Kamm and Steponkus, 1984). The reduction of water in the cell also increases the density of cytoplasmic components which increases molecular interactions. This can cause protein denaturation and membrane fusion (Hoekstra *et al*., 2001).

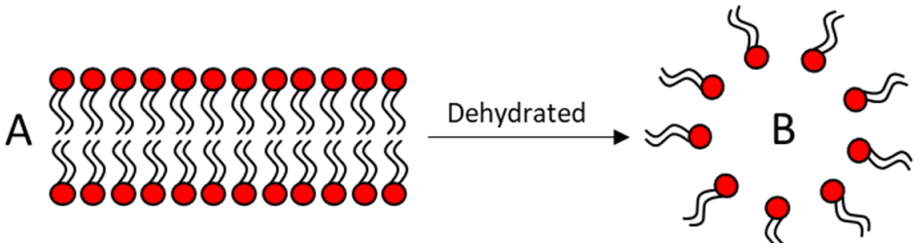

Figure 3. Schematic representation of the lamella before dehydration (A) and after dehydration (B). When dehydration occurs the lamella transitions from a linear plain (A) to hexagonal phase II (B).

Mechanical stress can be caused by a phenomenon known as expansion-induced lysis; a form of freeze-thaw injury to the cell membrane. Plasmolysis occurs upon losing osmotically active water to extracellular freezing due the disparity in water potential. When the temperature abates, thawed water travels down its concentration gradient back into the cell. Deplasmolysis is unable to take place after thawing as the sudden uptake of water occurs too rapidly to allow re-expansion of the cell and this results in the cell lysing (Dowgert and Steponkus, 1984). If lysis does not occur there may also be loss of osmotic responsiveness (LOR). This occurs

after the cell has shrunk and the damage prevents the cell from returning to its hydrated shape (Yamazakia *et al*., 2009).

Temperature is also responsible for regulating enzyme function. All enzymes have an optimal operating temperature where catalytic activity is maximised and a range of temperatures within which they are able to function. Exceed or drop below the functional threshold temperature and the enzyme can become denatured or incapacitated. Thus, as enzymes are essential for metabolic function a wide range of problems can ensue at low temperatures (Privalov, 1990). To prevent injuries during freezing temperatures, some plants go through a physiological process called cold acclimation. Cold acclimation is most common among plants from temperate and polar climates and is an essential survival strategy against freezing temperatures.

1.3 Cold Acclimation

Cold acclimation is a process whereby low temperatures (<4 °C but above freezing), can be used to prepare the plant for below-freezing temperatures. Cold acclimation reduces freezing injury, enabling plants to survive lower temperatures than would be possible without acclimating. Notably, upon induction of freezing temperatures, plants that cold acclimate display rapid alterations in gene expression (Guy *et al*., 1985). Analyses of transcript changes during cold acclimation have revealed a substantial number of important genes among which the *C-REPEAT BINDING FACTOR* (*CBF*) and *COLD RESPONSE* (*COR*) genes have been paramount in understanding the cold acclimation process. The CBF pathway is involved in many transcriptional responses which protect against freezing damage (Summarised in Figure 4). In rice, low temperatures are putatively sensed by alterations in the cell membrane which activate CHILLING-TOLERANCE DIVERGENCE 1 (COLD1), a heterotrimeric G protein which spans the plasma membrane and endoplasmic reticulum (ER) (Ma *et al*., 2015; Zhu *et al*., 2016). COLD1 interacts with its alpha subunit RICE G-PROTEIN ALPHA SUBUNIT 1 (RGA1) which upon phosphorylation, induces conformational changes in calcium ion channels in the plasma membrane. Influxes of calcium have also been observed in Arabidopsis following cold stress, although no membrane-based cold sensor has been identified (Knight and Knight, 2012). Calcium activates

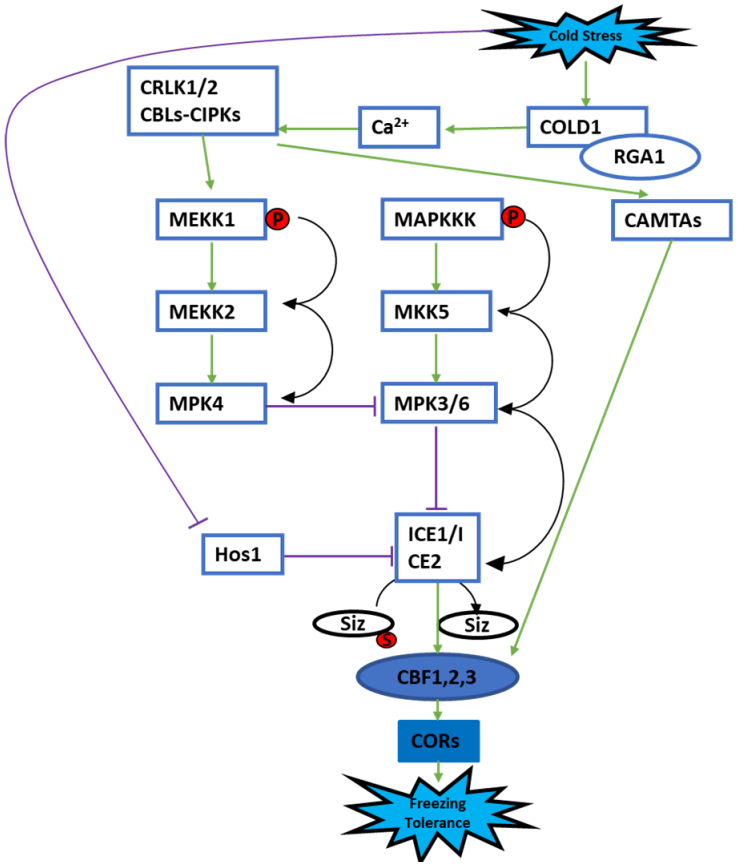

Figure 4. Molecular response following perception of cold stress in *Arabidopsis thaliana*. Green arrows indicate upregulation, purple blunt arrows indicate inhibition and black arrows indicate phosphorylation. For detailed summary and references of the cold response pathway refer to the section on cold acclimation. CHILLING-TOLERANCE DIVERGENCE 1 (COLD1), Rice G-protein α subunit (RGA1), CALCIUM/CALMODULIN-REGULATED RECEPTOR-LIKE KINASE (CRLK1/2), CALCINEURIN B-LIKE-CIPKs (CBLs-CIPKs), MITOGEN ACTIVATED PROTEIN KINASE KINASE KINASE 1 (MEKK1), MITOGEN ACTIVATED PROTEIN KINASE KINASE 2 (MKK2), MITOGEN ACTIVATED PROTEIN KINASE 4 (MPK4), INDUCER OF CBF EXPRESSION (ICE1/2), C-REPEAT BINDING FACTOR (CBF), COLD RESPONSE (CORs), HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE (HOS1), CALMODULIN BINDING TRANSCRIPTION ACTIVATOR (CAMTA).

CALCIUM/CALMODULIN-REGULATED RECEPTOR-LIKE KINASE (CRLK 1/2) which begins a kinase cascade where MITOGEN ACTIVATED PROTEIN KINASE KINASE KINASE 1 (MEKK1), MITOGEN ACTIVATED PROTEIN KINASE KINASE 2 (MKK2) and MITOGEN ACTIVATED PROTEIN KINASE 4 (MPK4) become phosphorylated respectively (Yang *et al*., 2010). MPK4 interacts with and inhibits MPK3/6, preventing the degradation of INDUCER OF CBF EXPRESSION 1 (ICE1) through ubiquitination (Zhu *et al*., 2016). ICE1 is a constitutively expressed MYC-like bHLH (beta-helix-loop-helix) transcription factor that binds to the *CANNTG MYC*-binding site present in the promoters of *CBF 1-3* (Chinnusamy, *et al*., 2003; Meshi and Iwabuchi, 1995). Both the calcium -dependent and -independent pathways are thought to be involved in sumoylation of ICE1 via SIZ proteins while simultaneously inhibiting ICE1 ubiquitination. The *CBF* genes encoded by Arabidopsis are *CBF1*, *CBF2* and *CBF3*, also referred to as *DEHYDRATION RESPONSE ELEMENT BINDING PROTEIN* 1,2 and 3 (*DREB1*, *DREB2* and *DREB3;* Stockinger *et al*., 1997 and Liu *et al*., 1998). The *CBF* genes are expressed within 15 minutes exposure of 4 °C. This expeditious expression emphasises their importance in regulating freezing tolerance and has made them a primary target of investigation for cold acclimation research (Gilmour *et al*., 1998 and Stockinger *et al*., 1997). *CBF* genes are located in an 8.7 kb region on chromosome 4 in a tandem array and encode proteins which are closely related to the APETALA2/ETHYLENE-RESPONSE ELEMENT-BINDING FACTOR (AP2/ERF) family of transcription factors.

CBF proteins bind to a cis-acting element, a conserved CCGAC sequence found in the promoter region of target genes (*COR* genes) known as the C-repeat (CRT) or DEHYDRATION RESPONSE ELEMENT (DRE) (Stockinger *et al*., 1997). *COR* genes are upregulated by CBFs within 2-3 h of cold exposure. Over 100 *COR* genes have been identified which make up the *CBF* regulon, yet the process by which freezing tolerance is achieved has not been fully elucidated. What is known is that the *CBF* regulon is responsible for the proliferation of cryoprotectant proteins which result in resistance to cold related injury (Hughes *et al*., 2013).

When the cold acclimation pathway is not active, ICE1 undergoes ubiquitination via HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE (HOS1) (Dong *et al*., 2006) and MPK3/6 which phosphorylates ICE1, resulting in proteasomal degradation. This prevents the expression of *COR* genes as *CBF* transcription is hindered. This was demonstrated using the mutant *ice1* which showed a significant reduction in resistance to freezing temperatures (Chinnusamy, *et al*., 2003; Dong *et al*., 2006 and Knight and Knight, 2012). Calcium-independent pathways also respond to low temperatures. COLD RESPONSIVE PROTEIN KINASE (CRPK1) is a plasma membrane- imbedded protein that responds directly to cold through putative membrane changes and acts as a negative regulator of CBF signalling.

CPRK1 interacts with 14-3-3 proteins which in turn inhibit the expression of *CBF*s (Shi *et al*., 2018). Although CBF s have a paramount role in cold induced protection, microarray analyses have revealed that the CBFs are responsible for regulating only 12% of the cold-responsive transcriptome in Arabidopsis (Fowler and Thomashow, 2002). CBF- independent pathways have also been identified which contribute to cold tolerance, including the ZAT12 regulon (Vogel *et al*., 2005). Cold responses in plants do not operate in isolation and have been shown to integrate with a number of other abiotic signalling pathways. Of these, light is particularly important. The integration of cold and light signalling is discussed below.

1.4 Light signalling

Light plays a crucial role in plant development. Not only is it the primary source of energy through photosynthesis but light signals also provide an information signal that can direct morphological and physiological responses. Light responses are governed by specific wavelengths, from UV-B (280-315 nm) to far red (700-750 nm). Different wavelengths of light are perceived by photoreceptors. In Arabidopsis, red (600-700 nm) and far red (700-750 nm) wavelengths are detected by a family of five phytochromes (Phy A-E). Blue light (400-500 nm) and UV-A (315-400 nm) are detected by phototropins (Phot1 and Phot2; Briggs *et al*., 2001), cryptochromes (Cry1 and Cry2; Cashmore *et al*., 1998) and zeitlupe family members (ZTL, FKF1 and LKP2; Takemiya *et al*. 2005; Kami *et al*., 2010). UV-B (280-315 nm) is detected by UV RESISTANCE LOCUS 8 (UVR8; Rizzini *et al*., 2011) (Fig 5).

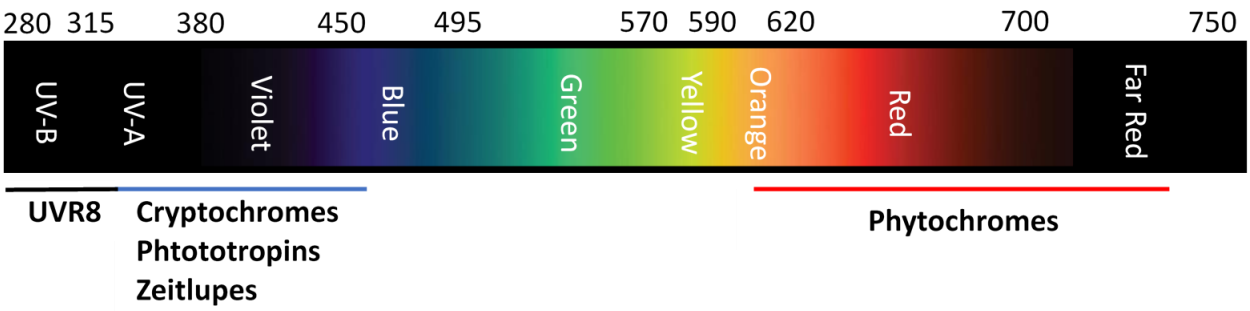

Figure 5. Light spectrum from UV-B to far red. Key photoreceptor activity ranges are indicated by horizontal bars

1.5 UV-B

UV-B is transmitted through the Earth's atmosphere and has the highest energy of all transmitted light at the Earth's surface. The high energy levels of UV-B make it potentially damaging and the higher the UV-B fluence, the higher the risk of plant damage. Ambient levels of UV-B are dependent upon multiple factors, including solar angle (time of day, season and latitude), ozone cover, altitude, shade and cloud cover (Jenkins, 2009). Since UV-B levels are so variable, a constant UV-B defence response is not required by plants. Investment in UV-B defence is therefore initiated and adjusted when necessary, thus optimising resource allocation.

When UV-B levels are high enough (>1 μM $m^{-2}s^{-1}$), UV-B radiation can damage plants through the production of ROS (Tong *et al*., 2008). This is the consequence of ionizing radiation interacting with water molecules within the organism, a process known as radiolysis. Free radicals are produced including ˙OH (Hydroxyl radical) and H^2O^2 (Hydrogen peroxide) which are highly reactive and interact with surrounding macromolecules including lipids, proteins and DNA (Esnault *et al*., 2010). Free radicals damage DNA through single strand and double strand breaks. Cyclobutene pyrimidine dimers (CPDs) account for 75% of UV-B-induced DNA damage (Takahashi *et al*., 2011). When damaged DNA is repaired, errors can also occur. These include miscoding and non-coding lesions within genes. If these mutations go unnoticed or unrepaired then the stability of the genome can be reduced, negatively affecting plant metabolism and survival (Gill and Tuteja, 2010).

To mitigate the damaging effects of UV-B, plants have evolved a UV-B signalling response pathway. This is initiated by UVR8 upon the detection of UV-B. UVR8 is the first reported photoreceptor in plants that is not dependent upon an external chromophore for light detection. UVR8 encodes a seven-bladed β-propeller protein which exists as a homodimer when inactive (Kliebenstein *et al*., 2002). Sensing UV-B is achieved via tryptophan residues located on the UVR8 molecule (W285 and W233 are the most prominent tryptophan residues involved with absorbance of UV-B). The residues exist as pairs which bind UVR8 as a homodimer through salt-bridge interactions between charged amino acids (O'Hara and Jenkins, 2012). When UV-B is absorbed by tryptophan residues, the interaction between UVR8 homodimers is disrupted. This allows monomerization to occur, initiating a signalling cascade (Rizzini *et al*., 2011). Monomerised UVR8 interacts with the E3 ubiquitin ligase, CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1), in a UV-B- dependent manner (Favory *et al*., 2009). Specifically, the UVR8 monomer and COP1 interact through the β-propeller, via the C27 domain (Yin *et al*., 2015). This interaction sequesters COP1 and prevents degradation of the basic zipper transcription factor, ELONGATED HYPOCOTYL 5 (HY5; von Arnim and Deng,

1994). UVR8 signalling is putatively positively regulated through SUPPRESSOR OF PHYTOCHROME A-105 (SPA 1-4) proteins which bind to the UVR8-COP1 complex and enhance the function of COP1 in UV-B (Huang *et al*., 2013). FAR-RED ELONGATED HYPOCOTYL 3 (FHY3), a transposase transcription factor, and HY5 are involved in upregulating COP1 (Huang *et al*., 2012). HY5 binds to and regulates thousands of genes (Lee *et al*., 2007) and, together with its homologue, HY5 HOMOLOG (HYH) promote UV-B signalling (Ulm *et al*., 2004). HY5 is known to bind to the promoter of MYB12 which activates expression of UV-B induced genes. These include *CHALCONE SYNTHASE* (*CHS*) and *CHALCONE ISOMERASE* (*CHI*) which are involved in flavonoid biosynthesis (Stracke *et al*., 2010, Figure 6).

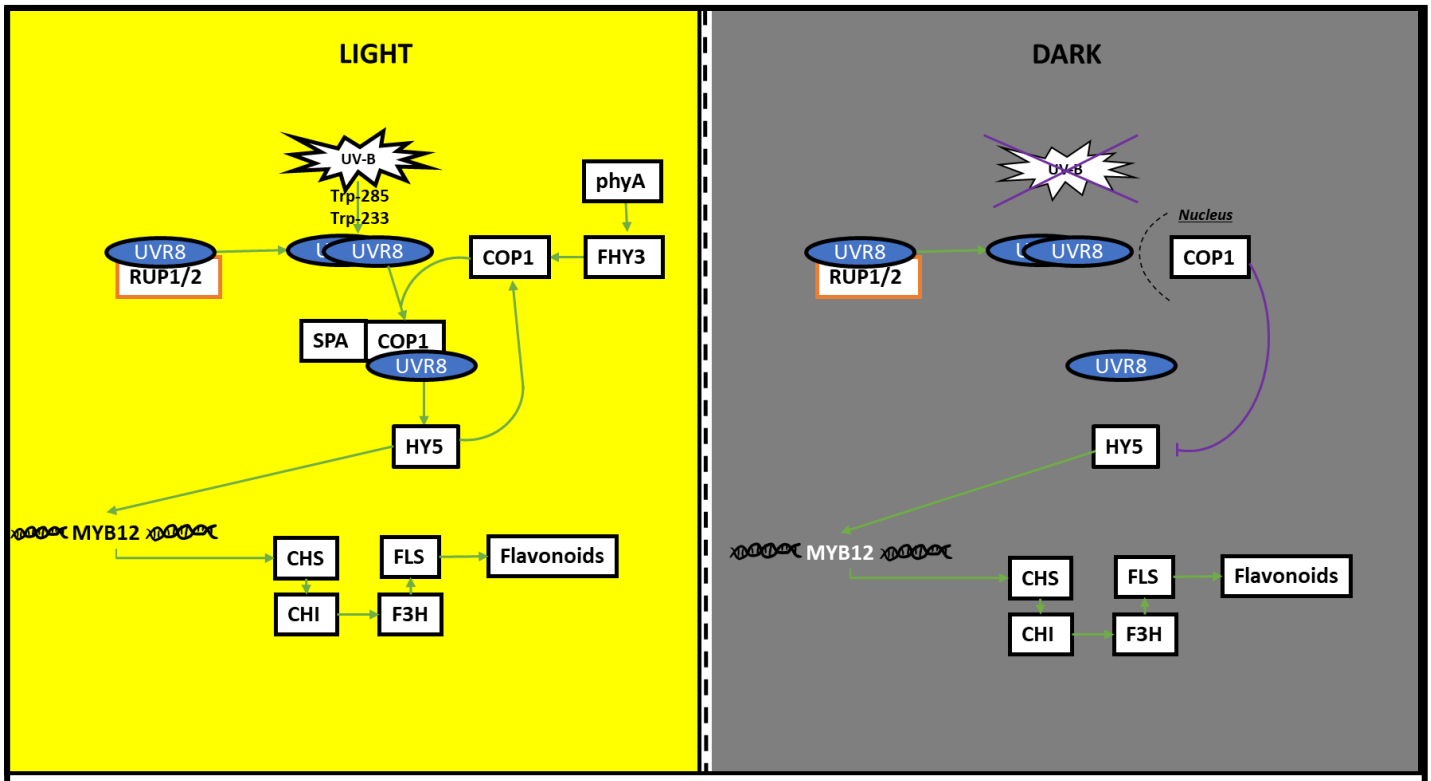

Figure 6. Plant UV-B response pathway in the presence of light (left) and in the dark (right). Green arrows indicate upregulation, purple blunt arrows indicate inhibition. For a detailed summary of the pathway and references refer to the section on light and UV-B signalling. UV RESISTANCE LOCUS 8 (UVR8), REPRESSOR OF UV-B PHOTOMORPHOGENESIS (RUP1/2), CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1), SUPRESSOR OF PHYTOCHROME A-105 (SPA), ELONGATED HYPOCOTYL 5 (HY5), Phytochrome A (PhyA), FAR-RED ELONGATED HYPOCOTYL 3 (FHY), CHALCONE SYNTHASE (CHS), CHALCONE ISOMERASE (CHI), FLAVONE 3 HYDROXYLASE (F3H), FLAVANOL SYNTHASE (FLS).

1.6 Light-regulation of plant freezing tolerance

Different abiotic stresses can cause damage to plants through similar mechanisms. For example, drought, salinity high temperature and freezing stress are all associated with dehydration of the plant. It is therefore not surprising that crosstalk exists between abiotic signalling pathways. Light plays an important role in regulating freezing tolerance in plants. Over-expression of PhyA in hybrid aspen has been demonstrated to prevent cold acclimation (Olsen *et al*., 1997), and in Arabidopsis, PhyB has been shown to be involved in repressing the CBF pathway in long days, via the transcription factors PHYTOCHROME INTERACTING FACTOR 3, 4 and 7 (PIF) (Lee and Thomashow, 2012; Jiang *et al*., 2017). Crosstalk between light signalling and the circadian clock exists in plant cold signalling. The circadian clock plays an important role in regulating *CBF* expression and the phytochrome photoreceptors entrain the clock (Somers *et al*., 1998). Kidokoro *et al*. (2017) have suggested that plants differentiate between cold temperatures based upon the speed of cold induction. Temperatures that decrease gradually are associated with seasonal changes in temperate climates and result in the expression of *CBF1* which is modulated by the circadian clock components CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY). Furthermore, Franklin and Whitelam (2007) demonstrated that low red to far-red ratio (low R:FR) light which is characteristic of canopy shade was capable of inducing *CBF* expression and freezing tolerance at temperatures higher than what would be required for cold acclimation to take place.

Further crosstalk between light and cold signalling in Arabidopsis may occur through *HY5.* This encodes a bZIP TF that acts as a central regulator of light and temperature signalling due to the thousands of genes it recognises through the Z-box (Catalá *et al*., 2011; Zhang 2011). *HY5* is positively regulated at the transcriptional level by light through the phytochromes and in the absence of light HY5 is degraded by COP1. The importance of HY5 in UV-B protection was highlighted by Brown *et al*. (2005) who showed that *hy5* in Arabidopsis displayed higher levels of damage in the presence of UV-B than WT controls. HY5 is involved in the upregulation of flavonoids through MYB12 which protect plants from UV-B damage and may protect plants from low temperatures (Stracke *et al*., 2010; Schulz *et al*., 2016) (Discussed in chapter 4). Catalá *et al*. (2011) showed that HY5 acts through the Z-box to mediate the induction of 10% of all the Arabidopsis cold induced genes. They additionally demonstrated that both HY5 and COP1 were focal points of crossover between the cold acclimation and light signalling pathways. As UV-B stabilises and up-regulates HY5 (Ulm *et al*., 2004), it is possible that UV-B may enhance freezing tolerance.

1.7 UV-B-regulation of plant freezing tolerance

Growth inhibition is a common response to both UV-B and cold exposure and involves regulation of the growth hormone gibberellic acid (GA; Achard *et al*., 2008; Hayes *et al*., 2014). CBF1 and HY5 are both involved in upregulating *GA2ox* (GIBBERELLIC ACID 2 oxidase) which degrades GA (Thomas *et al*., 1999). GA is thus prevented from degrading growth-repressing DELLA proteins which are upregulated in the cold (Tyler *et al*., 2004, Piskurewicz, and Lopez-Molina, 2009) and UV-B (Hayes *et al*., 2014). DELLAs repress growth by inhibiting the accumulation and activity of PIF transcription factors (de Lucas *et al*., 2008; Feng *et al*., 2008; Li *et al*., 2016) which promote growth via the up-regulation of auxin biosynthesis (Franklin *et al*., 2011). Growth inhibition has been suggested to be an adaptive response which allows resources to be diverted towards protection in adverse conditions. It may therefore promote survival under cold and UV-B stress (Tong *et al*., 2008) (Figure 7). Further evidence of crosstalk between UV-B and low temperature signalling has been provided by Dunning *et al*. (1994), who exposed Rhododendron leaf discs to UV-B and recorded increased freezing tolerance. Yang *et al*. (2007), working with *Triticum aestivum* seedlings, also showed that exposure to UV-B increased freezing tolerance through upregulation of ROS scavenging enzymes.

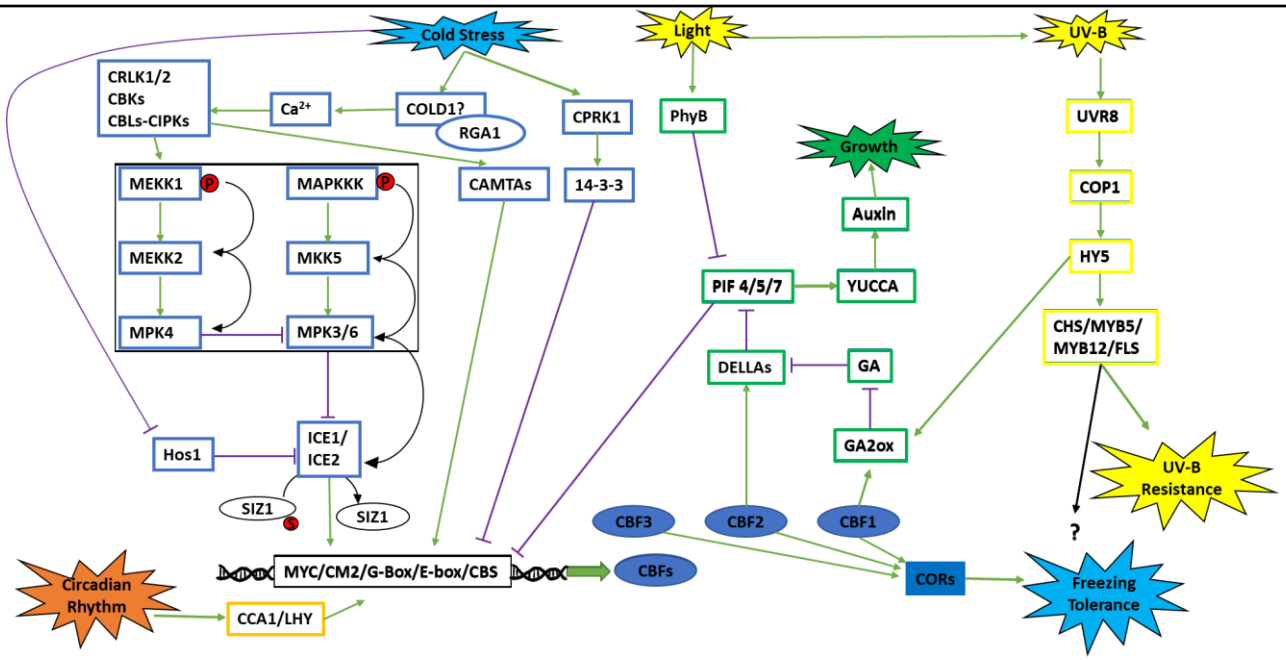

Figure 7. Integration of the Arabidopsis cold stress pathway (indicated by blue boxes) and UV-B stress pathway (indicated by yellow boxes) showing points of potential crossover between the two pathways. Green boxes indicate the genetic pathway that leads to growth and the integration of the circadian rhythm is indicated by orange boxes. Green arrows indicate upregulation, purple blunt arrows indicate inhibition, bent black arrows indicate phosphorylation, black arrows followed by a question mark indicate a putative link between stress pathways. Please refer to primary text for abbreviations and a more in-depth discussion of the pathways.

1.8 Project aims

Despite evidence suggesting crosstalk between UV-B and low temperature signalling, the impact of UV-B on Arabidopsis cold acclimation and freezing tolerance has not been tested. The aims of this project are therefore to investigate whether UV-B supplementation can enhance Arabidopsis freezing tolerance and whether crosstalk between cold and UV-B signalling exists. This will be addressed by the following questions:

1. Does UV-B supplementation affect the survival of Arabidopsis plants subjected to freezing temperatures?
2. Does UV-B enhance the low temperature-induction of *COR* genes?
3. Do UV-B and low temperature signals interact to control flavonoid accumulation?

CHAPTER 2: MATERIALS AND METHODS

2.1 Seed stocks and Growth Conditions

The mutant lines used in this study in the Columbia-0 (Col-0) background were *uvr8-6* (Favory *et al*., 2009) *tt4* (Winkel-Shirley *et al*., 1995) and *tt7* (Winkel-Shirley *et al*., 1995). Seed sterilisation was adapted from the Podar (2013) protocol. Seeds were surface sterilised by submerging seeds in 70% ethanol (EtOH) for 10 min. They were then washed twice in deionized water before placing either onto agar or compost.

Plants were grown on compost, 3 parts Compost (Sinclair all-purpose growth medium, William Sinclair Horticultural Ltd, Lincoln, UK): 1 part Sand (Horticultural Silver Sand, Melcourt Garden and Landscape, Tetbury, UK). Seeds were sown onto a compost filled petri dish (50 x 18 mm) or onto agar media (Murashige and Skoog (MS) medium, 1% w/v sucrose, 0.6% w/v agar, pH 5.8) according to Podar (2013) and then transplanted onto soil.

Seeds were stratified for 72 h in the dark at 4 °C. Following this, compost plates were placed into a tray and covered with perforated clingfilm lids to allow the transmittance of UV-B and maintenance of moisture and humidity. Trays were then placed under white light (PAR 70 µmol $m^{-2}s^{-1}$) (White light bulbs used: Philips master TL-D 36W/840, Amsterdam, Netherlands) in a growth cabinet (MC1600E, Snijders Scientific, Tilburg, Netherlands) maintained at 20 °C, 70% relative humidity (RH) and a light/dark cycle of 16 h/8 h respectively for 72 h to allow germination. Following germination, plants designated for UV-B treatment were placed under a mixture of UV-B (1 µmol $m^{-2}s^{-1}$) and white light (70 µmol $m^{-2}s^{-1}$) (Appendix, figure S1, S2 and S3). UV-B was provided by narrowband tubes (TL 40W/01 – RS, Philips, Amsterdam, Netherlands). UV-B and PAR levels were measured using an Ocean Optics Flame Spectrometer (Ocean Optics, Oxford, UK) in association with Ocean View 1.6.7 software Plants were watered using de-ionized water (Purite water system, SUEZ water, Thame, UK) every 72 h using a wash bottle until the compost was damp. After 14 days growth had occurred, plants designated for cold acclimation were placed into a separate growth cabinet (Jumo dTRON 304, Snijders Scientific, Tilburg, Netherlands) fitted with blue and red LEDs at 4 °C for 24 h (Appendix figure S4 and S5). PAR and UV-B levels were replicated in the cold acclimation chamber and plants received continuous red and blue light for the duration of cold acclimation period.

2.2 Whole plant freezing tolerance assay

The number of plants designated for freezing tolerance assay were counted prior to freezing to allow percentage survival to be calculated. Following a 24 h cold acclimation treatment, half of each genotype were subjected to freezing stress; 1 h at 0 °C followed by -6 °C for 24 h in darkness. All plants were then returned to initial growth conditions and allowed to recover for a further 14 d. Percentage survival was assessed by counting the number of plants still alive at this timepoint divided by the total number of plants at the start of the test. Photographs of plants following recovery were taken using a Samsung Galaxy Note 8 (Samsung, Seoul, South Korea).

2.3 Quantitative Polymerase Chain Reaction

2.3.1 RNA extraction

The relative transcript abundance of *COR15a*, *COR47* and *CHS* was recorded following a 24 h cold acclimation treatment. Aerial plant material was removed using scissors and tweezers sterilised with ethanol and RNase ZAP (Sigma Aldrich, Missouri, USA). Plant samples were placed into a microcentrifuge tube containing two 3 mm steel ball bearings and submerged in liquid nitrogen before homogenisation using a tissue lyser (Tissue lyser 2, Qiagen, Hilden, Germany) for 5 min at 30 Hz. RNA was extracted using a Spectrum™ Plant Total RNA Kit (STRN250-1KT, Sigma-Aldrich, Missouri, USA) according to the manufacturer's protocol. Genomic DNA was removed from each sample using the Amplification Grade DNAse I kit (AMPD1, Sigma-Aldrich, Missouri, USA), according to the manufacturer's instructions.

2.3.2 cDNA synthesis

RNA quantity was checked using a Nanodrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). RNA was then diluted to 1 µg using nuclease free water and cDNA synthesis performed using the Applied Biosystems High Capacity cDNA Reverse Transcription kit (4368814, Thermo Fisher Scientific, Massachusetts, USA).

2.3.3 qPCR

A cDNA dilution series was first performed to check primer efficiency (Appendix figure S6). cDNA results from qPCR were analysed using 2-ΔΔCt algorithm (Pfaffl, 2001), and data was normalised to *PP2A* expression. *PP2A* was selected as a reference gene due to its invariance under abiotic stress in shoot development (Czechowski *et al*., 2005). *PP2A* was further compared to two other reference genes, *UBC21* and *PIP41*, to check for conformity and reliability in results between refences genes (Appendix figure S7).

qPCR was performed using a 2-step thermal profile and dissociation/melt curve (Agilent Technologies Stratagene Mx3005P, California, USA) and qPCR analysis was performed using MxPRO software (Agilent Technologies Stratagene Mx3005P, California, USA).

Table 2. Primer name and sequences

Primer Name	**Primer Sequence**
COR15a Forward	GGC CAC AAA GAA AGC TTC AG
COR15a Reverse	CTT GTT TGC GGC TTC TTT TC
COR47 Forward	AGC TTC ACC GAT CCA ACA GCT CTT C
COR47 Reverse	CGG GAT GGT AGT GGA AAC TGG
CHS Forward	ATC TTT GAG ATG GTG TCT GC
CHS Reverse	CGT CTA GTA TGA AGA GAA CG
PP2A Forward	GTT CTC CAC AAC CGC TTG GT
PP2A Reverse	TAA CGT GGC CAA AAT GAT GC

2.4 Pigment quantification

Plants used for pigment quantification were grown for 28 days to allow Arabidopsis leaves to grow large enough for analysis using a Dualex spectrometer (Dualex Scientific, Centre Universitaire Pans-Sud, France). The Dualex allows the measurement of chlorophyll anthocyanin and flavonoid levels to be taken over successive days from the same plant unlike a chemical bioassay and therefore allows a change over time to be measured from the same leaf. The 1st leaf of the rosette was selected for pigment analysis and a piece of cotton was tied around the petiole to ensure the same leaf was measured over subsequent days. Plants were subjected to the same growth conditions except during cold acclimation. A 72 h cold acclimation period was used to allow time for flavonoid production and measurement. 3 adaxial and 3 abaxial readings were taken at the beginning of the experiment and from the same leaf every 24 h for 72 h.

2.5 Statistics

A three-way ANOVA was used to assess statistically significant differences between treatments and the interaction between treatments in the freezing tolerance assays. Pairwise analysis was conducted using a Tukey post-hoc analysis. Kruskal Wallis tests were performed for all qPCR data sets as data was not normally distributed. For all statistical tests α was set to 0.05. Normality was assessed using a Levine's test. To increase normality the percentage data was arcsine transformed to allow an ANOVA to be performed. All statistical analyses were performed using SPSS (IBM, New York, USA).

2.6 Thin Layer Chromatography

Flavonol glycosides were extracted and analysed using thin layer chromatography (TLC) adapted from the protocol by Stracke *et al*. (2010). 100 mg of aerial leaf tissue was harvested and placed in microcentrifuge tubes containing two 3 mm steel ball bearings and placed into liquid nitrogen. Samples were vortexed, homogenising the leaf tissue and 100 µl 80% (v/v) of MeOH was then added. Tubes were incubated at 70 °C for 15 min and then centrifuged for 10 min at 13200 rpm in an Eppendorf 5415R centrifuge (Eppendorf, Hamburg, Germany). Supernatants were extracted and 8 µl of each spotted onto HPTLC silica gel 60 glass plates (Millipore UK ltd, Hertfordshire, UK). Plates were placed in a closed glass container with a prepared mobile phase (ethyl acetate, formic acid, acetic acid and water, 100:26:6:12 v/v respectively) for 30 min. These were then removed and air dried for 15 min. 2 ml of 1% (w/v) 2,3-dibromopropanal (DPBA) in MeOH was sprayed onto the plates three times leaving 2 min between spraying. 2 ml of 5% (w/v) PEG 4000 (AppliChem, Darmstadt, Germany) in MeOH was then applied similarly. Plates were air dried for 15 min and visualised under UV light (365 nm). Flavonol glycoside-DPBA derivatives fluoresce under UV and the flavonols have been assigned different colours for qualitative analysis, Quercetin is represented by orange on the TLC plate and kaempferol is represented by green (Stracke *et al*., 2010).

CHAPTER 3: UV-B MAY ENHANCE COLD ACCLIMATION IN ARABIDOPSIS

3.1 Introduction

Plants have adapted to freezing temperatures through a multitude of physiological and molecular adaptations. An adaptation widely employed by temperate plants is cold acclimation (CA). Using light and temperature cues from the environment, plants can monitor seasonal changes and anticipate the approaching winter. When temperatures drop to around 4 °C, genes involved in cold acclimation are significantly upregulated. This involves the *CBF* regulon which is activated by MYC-like bHLH transcription factors, ICE 1 and ICE 2. The CBF proteins bind to a cis-acting element, a conserved *CCGAC* sequence found in the promoter region of target genes (*COR* genes) known as the C-repeat (CRT) or DEHYDRATION RESPONSE ELEMENT (DRE) (Stockinger *et al*., 1997) (Figure 4). Among the *COR* genes that are upregulated during cold acclimation is *COR15a*, an important marker of cold acclimation. Low temperatures promote the transcription of *COR15a* in the nucleus, and the hydrophilic polypeptide product is then transported to the chloroplast stroma (Lin and Thomashow, 1992; Nakayama *et al*., 2007; Candat *et al*., 2013). The importance of *COR15a* in protecting plants during freezing temperatures was demonstrated by Artus *et al*. (1996) who showed that chloroplasts and protoplasts were more freezing tolerant in transgenic plants constitutively expressing *COR15a*. Complementing this study, Nakayama *et al*. (2007) demonstrated that enzymes within the chloroplast are protected by *COR15a*, which prevents freeze-induced inactivation. However, contrary to this Thalhammer *et al*. (2014) analysed *COR15a* overexpression lines and found no improvement in the protection of several enzymes at low temperature. They instead suggest that enzyme protection during low temperatures is due to other factors involved in cold acclimation and not *COR15a*. What has been clearly demonstrated is that *COR15a* stabilises lipids in the membrane of the chloroplast during low temperatures preventing solute leakage and thus protecting the chloroplast during low temperatures (Thalhammer *et al*., 2014).

Another important *COR* gene upregulated in low temperatures is *COR47*. *COR47* encodes a hydrophilic boiling soluble polypeptide which belongs to the LATE EMBRYOGENESIS ABUNDANT II (LEA II) protein family (Gilmour *et al*., 1992). These proteins are abundant in the final stages of seed development allowing seeds to be highly drought resistant. LEA II proteins also known as dehydrins are proteins that are expressed under environmental conditions that result in cellular dehydration. This includes low temperatures, high temperatures, drought and high salinity (Wisniewski *et al*., 1996; Kosová *et al*., 2007). Freezing temperatures cause cellular dehydration through the formation of ice crystals in the extracellular space. This reduces water potential outside the cell causing osmotically active

water to move from within the cell to the extracellular space where it then freezes making it biologically unavailable (Hansen and Beck, 1988; Chang, 1999; Thomashow, 1999). Although the exact role of dehydrins is yet to be confirmed they have been proposed to act as chaperones that interact with and protect proteins from denaturing during cellular dehydration (Koag *et al*., 2003). Evidence also exists that demonstrates their importance in resisting cold stress. Xing *et al*. (2011) showed that higher levels of dehydrins correlate to lower levels of injury during freezing temperatures as measured by electrolyte leakage. Investigating both *COR15a* and *COR47* transcript abundance may reveal if UV-B can regulate freezing tolerance through dehydration resistance (*COR47*), membrane protection (*COR15a*) or both response pathways.

As cold acclimation is a very important process in protecting plants against freezing temperatures, understanding if this process is enhanced by UV-B would be a good indicator of whether there is potential for UV-B to enhance freezing tolerance. Therefore, if UV-B enhances *COR15a* or *COR47* transcript abundance, UV-B may be acting to regulate cold acclimation. This was analysed by qPCR. The *uvr8-6* mutant was included to identify whether any potential effects of UV-B were mediated through UVR8. Whole plant freezing tolerance assays were also used to identify whether UV-B supplementation could enhance freezing tolerance (Thorlby *et al*., 2003).

3.2 Results

Both genotypes (WT and *uvr8-6*) were grown at 20 °C for 2 weeks following germination. 50% of the plants were grown with UV-B supplementation (1 µmol $m^{-2}s^{-1}$). 50% of plants grown under UV-B and 50% of plants grown in the absence of UV-B were cold acclimated for 24 h at the end of the 2-week growth phase in continuous red and blue light (treatment groups: UV-B cold acclimated (CA), UV-B non-cold acclimated (non-CA), no-UV-B CA and no-UV-B non-CA). Aerial leaf tissue was harvested for RNA extraction and qPCR analysis from Arabidopsis WT and *uvr8-6* plants from which *COR15a* and *COR47* relative transcript abundance were calculated. For whole plant freezing tolerance assays after the cold acclimation phase all plants were subjected to a -6 °C freezing treatment for 24 h and a 2-week recovery period.

3.2.1 UV-B and cold acclimation influence COR15a expression

qPCR was used to assess the ability of Arabidopsis to cold acclimate with and without supplementary UV-B and with and without a cold acclimation treatment (Figure 8). Plants receiving cold acclimation showed *COR15a* expression levels many times greater than non-cold acclimated treatments. Cold acclimated WT plants treated with UV-B displayed over a two-fold increase in *COR15a* transcript abundance compared with UV-B untreated controls. This trend was not evident in *uvr8-6* mutants as there was high variation in the data. Together these data suggest that cold acclimation treatment was successful and further show that UV-B supplementation when combined with cold acclimation causes a noticeable increase of *COR15a* transcript abundance. The role of UVR8 in this process could not be determined due to variation between biological repeats. UV-B had no noticeable effect on *COR15a* transcript abundance in non-cold acclimated WT and *uvr8-6* plants. Despite these trends, A Kruskal-Wallis test used to statistically test whether *COR15a* transcript abundance differed between groups showed no significant differences. This may reflect the small replicate numbers used (n = 3).

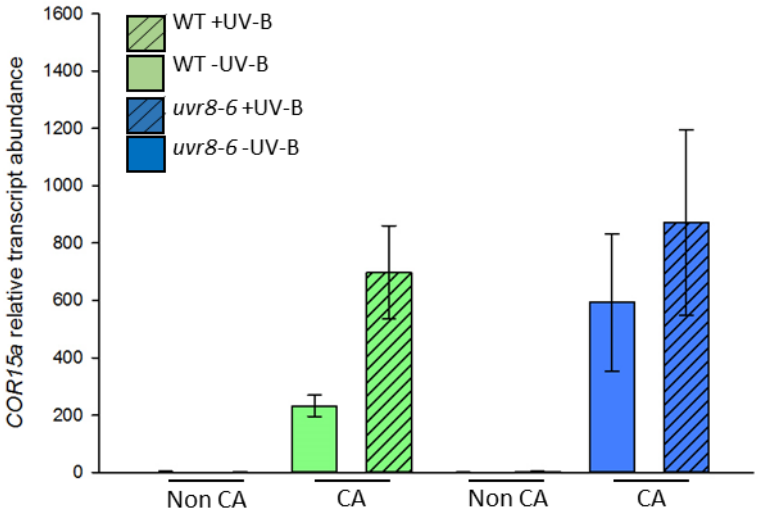

Figure 8. Relative transcript abundance of *COR15a* in 2- week-old Arabidopsis seedlings following cold and UV-B treatments. 50% of all plants were exposed to UV-B (1 µmol $m^{-2}s^{-1}$) during growth phase. Before leaf tissue was collected for RNA extraction 50% of plants in each treatment were cold acclimated for 24 h at 4 °C in continuous light ± UV-B. Non-CA = non-cold-acclimated, CA = cold-acclimated; striped columns indicate exposure to UV-B; green columns = WT, blue columns = *uvr8-6*. n = 3, SE bars.

3.2.2 UV-B and cold acclimation influence COR47 expression

COR47 transcript abundance was analysed using qPCR as it is an indicator of response to cellular dehydration caused by low temperatures. *COR47* transcript abundance was enhanced by cold acclimation treatment in WT and *uvr8-6* plants. Although there is high variation in the data, UV-B enhanced *COR47* transcript abundance in the cold-acclimated WT and *uvr8-6* plants. UV-B had no noticeable effect on *COR47* transcript levels in the non-cold acclimated WT and *uvr8-6* plants. A Kruskal-Wallis test was used to statistically test whether *COR47*

transcript abundance differed between groups. No significant differences were reported but this, again may reflect the small numbers of biological replicates used (n = 3) (figure 9).

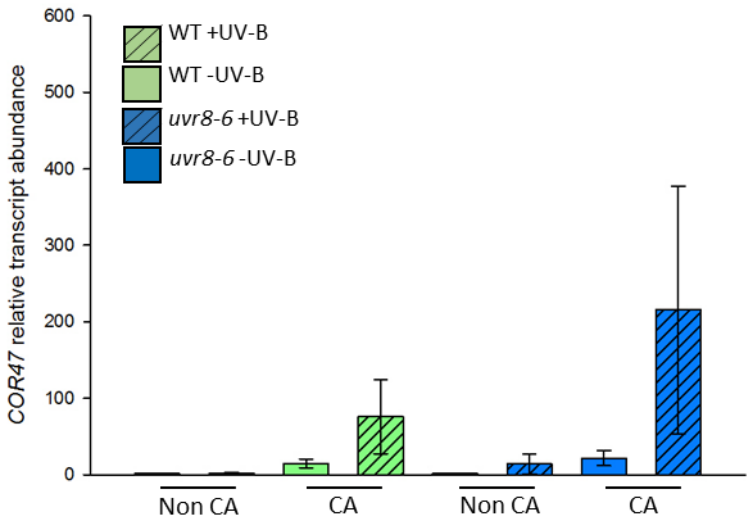

Figure 9. Relative transcript abundance of *COR47* in 2- week-old Arabidopsis seedlings following cold and UV-B treatments. 50% of all plants were exposed to UV-B (1 µmol $m^{-2}s^{-1}$) during growth phase. Before leaf tissue was collected for RNA extraction 50% of plants in each treatment were cold acclimated for 24 h at 4 °C in the light ± UV-B. Non-CA = non-cold acclimated, CA = cold acclimated; striped columns indicate exposure to UV-B; green columns = WT, blue columns = *uvr8-6*. n = 3, SE bars.

3.2.3 UV-B has no discernible impact upon freezing tolerance

Percentage survivability was measured in WT and *uvr8-6* plants after a -6 °C freezing treatment for 24 h and then a subsequent 2-week regrowth phase. To investigate the effect of temperature, UV-B and genotype on survivability, a 3-way ANOVA was performed. This showed that there was a statistically significant effect on survivability in temperature treatment [$F(1,23) = 3366.750$, $P < 0.001$]; UV-B treatment [$F(1,23) = 70.083$, $P < 0.001$]; and genotype [$F(1,23) = 44.083$, $P < 0.001$]. Statistically significant interactions between variables were also identified, temperature and UV-B [$F(1,23) = 70.083$, $P < 0.001$]; UV-B and genotype [$F(1,23) = 52.083$, $P < 0.001$]; temperature and genotype [$F(1,23) = 44.083$, $P < 0.001$]; genotype, temperature and UV-B [$F(1,23) = 52.083$, $P < 0.001$]. A Tukey post hoc analysis was performed to analyse which treatments were statistically significant. UV-B had no significant impact on survivability in WT plants but decreased survivability in *uvr8-6* mutants ($P < 0.001$). Cold acclimated WT plants treated with UV-B displayed significantly higher survivability than *uvr8-6* controls ($P < 0.001$; figures 10 and 11). Collectively, these data show that the cold acclimation treatment enhances survivability in WT and *uvr8-6* plants but UV-B supplementation has no noticeable impact on survival in this assay.

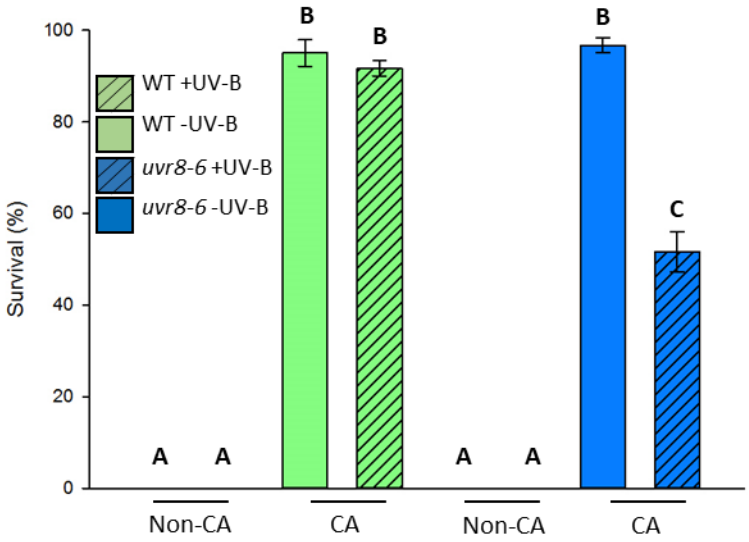

Figure 10. Percentage survival of 2-week-old Arabidopsis seedlings treated with cold and UV-B, following a 24 h -6 °C freezing treatment. 50% of all plants were exposed to UV-B (1 µmol $m^{-2}s^{-1}$) during the growth phase. Prior to the freezing treatment, 50% of plants in all treatments were cold acclimated for 24 h at 4 °C in the light ± UV-B. After the freezing treatment a 2-week regrowth phase was allowed before calculating percentage survival. Different letters indicate statistically significant differences between treatments (P <0.05). Non-CA = non-cold-acclimated, CA = cold-acclimated; striped columns indicate exposure to UV-B; green columns = WT, blue columns = *uvr8-6*. n = 60, SE bars.

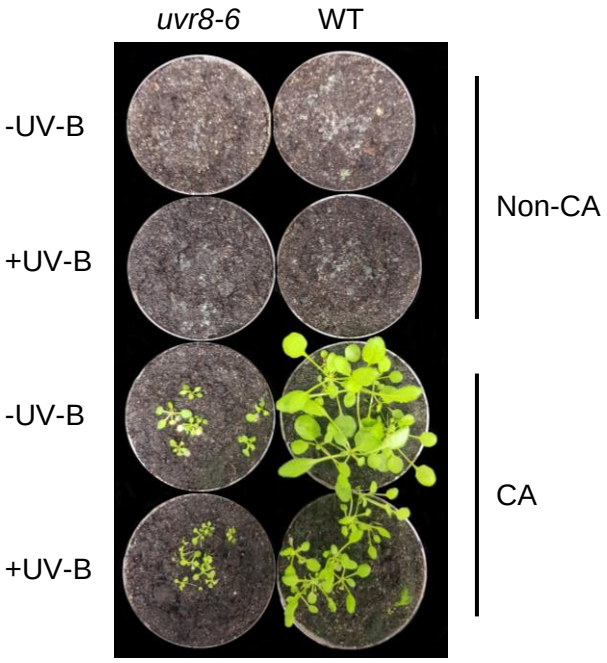

Figure 11. Photograph of cold- and UV-B-treated plants 2 weeks after being subjected to a -6° C freezing treatment. Treatment conditions were cold-acclimated (CA), non-cold-acclimated (non-CA) with UV-B (+UV-B) and without UV-B (-UV-B). Genotypes used were WT (Col-0), *uvr8-6*.

3.3 Discussion

As cold acclimation is an extremely important process in protecting plants against freezing temperatures, transcript abundance of the gene *COR15a* was selected as a proxy measure of freezing tolerance potential as it is an indicator of cold acclimation (Artus *et al*., 1996). Increased *COR15a* transcript abundance after 24 h cold acclimation has previously been described, which is why this timepoint was chosen for analysis (Franklin, and Whitelam, 2007). Cold-acclimated plants all showed increased transcript abundance compared with the non-cold- acclimated plants. This suggested that cold acclimation was successful in these experimental conditions and that low non-freezing temperatures are required to achieve cold acclimation. The presence of UV-B enhanced *COR15a* transcript abundance in cold acclimated WT plants, while trends were less clear in *uvr8-6* plants (figure 8). This suggests that UV-B and cold acclimation are acting synergistically, indicating crosstalk between UV-B and cold acclimation signalling pathways. However, the mechanism by which UV-B acts to enhance *COR15a* expression is currently unknown. The *uvr8-6* mutant line was used to understand if UV-B enhanced transcript abundance through a UVR8-dependent manner. Although there is an overall increase in *COR15a* transcript abundance in *uvr8-6* cold-acclimated and UV-B-treated plants compared with UV-B untreated controls there was high variation in the *uvr8-6* data which prevented any conclusions being made regarding the involvement of UVR8 (figure 8). More replicates would be required to reduce the variation and increase statistical power.

Similar to *COR15a*, *COR47* is also a marker of cold acclimation but is a dehydrin involved with preventing cellular dehydration. Cold acclimation treatments in both WT and *uvr8-6* plants increased *COR47* transcript abundance when compared with non-cold- acclimated groups. This result is concurrent with existing literature (Viswanathan and Zhu, 2002) and highlights the importance of *COR47* in the cold acclimation response. Despite high variation between biological repeats, data from both WT and *uvr8-6* plants indicated that in the presence of cold and UV-B, *COR47* transcript abundance was increased when compared to cold treatment alone (figure 9). Catalá *et al*. (2011) showed that the transcription factor HY5 binds to the Z box and is responsible for activating at least 10% of all cold inducible genes. Similarly, enhanced expression of *COR15a* and *COR47* under UV-B and cold acclimation could be explained by the activity of a presently unidentified transcription factor produced under these conditions that binds to an LTRE on the *COR* genes to enhance *COR* expression (Yamaguchi-Shinozaki and Shinozaki, 1994).

Consistent with published reports (Gilmour *et al*., 1988), cold acclimated groups showed a statistically significant higher percentage survival than non-cold acclimated groups. The non-

cold acclimated groups all had 0% survival, making it impossible to infer the effect of UV-B. Although transcript abundance analysis results from *COR15a* and *COR47* both suggest a role for UV-B enhancing cold acclimation (figure 8 and 9), the whole plant freezing tolerance assay showed no evidence of this (figure 10). Contrary to evidence posited by Dunning *et al*., (1994) and Yang *et al*., (2007), showing that freezing tolerance of Rhododendron and *Triticum aestivum* increased in the presence of UV-B, no statistical difference in survivability was found between UV-B treated and UV-B untreated Arabidopsis in this study. It is possible that with a more sensitive assay, an enhancement of freezing tolerance may be observed in UV-B-treated Arabidopsis plants. To assess the effects of UV-B in this assay, some survival in the non-cold acclimated groups would be required. To resolve this a lower freezing treatment could be administered to reduce the severity of cold stress. Survivability was very high in the cold acclimated groups, near 100% in WT plants treated with and without UV-B and near 100% in the UV-B untreated *uvr8-6* plants (figure 10). In combination with adjusting the freezing treatment, a shorter cold acclimation period could be used to decrease the survivability in the cold acclimated groups. These changes may increase the sensitivity of the assay and allow an analysis of any effects UV-B may be having on survivability. Alternatively, an electrolyte leakage assay could be used, this is an established method for measuring freezing tolerance in plants (Uemura *et al*., 2006; Hemsley *et al*., 2014).

Interestingly, in cold acclimated *uvr8-6* mutants, UV-B reduced survivability (figure 10). It is likely that these plants were exposed to a double stress. The *uvr8-6* genotype is deficient in the UVR8 photoreceptor known to be involved in sensing and responding to UV-B. In particular, UVR8 up-regulates protective compounds, such as flavonoids, involved in preventing UV-B-induced damage (Favory *et al*., 2009; Satio *et al*., 2013). Although the fluence rates of UV-B used here are generally considered low and non-stressful (1 µmol $m^{-2}s^{-1}$) (Tong *et al*., 2008) it may be that in combination with receiving a freezing treatment (-6 °C for 24 h) this level of UV-B becomes stressful.

Together, these data presented in this chapter suggest that UV-B and cold acclimation in combination enhance the transcript abundance of at least a proportion of *COR* genes. Although whole plant freezing tolerance assays were unable to confirm whether UV-B could enhance plant survival during freezing temperatures this could result from limitations in assay sensitivity.

CHAPTER 4: THE ROLE OF FLAVONOIDS IN UV-B AND LOW TEMPERATURE SIGNALLING CROSSTALK

4.1 Introduction

At low fluence rates (<1 µmol $m^{-2}s^{-1}$), UV-B is a photomorphogenic signal which regulates hypocotyl elongation (Ballaré and Barnes, 1995; Ros and Tevini, 1995; Hayes *et al*., 2014) and cotyledon expansion (Boccalandro *et al*., 2001). At higher fluence rates (>1 µmol $m^{-2}s^{-1}$), UV-B can damage plants through the production of ROS. These free radicals can damage macromolecules such as lipids, DNA and proteins (Esnault *et al*., 2010). Plants respond to this stress by depositing flavonoids in their epidermis which are capable of absorbing UV-B wavelengths (Caldwell *et al*., 1983). Flavonoids are ubiquitous throughout the plant kingdom and are considered secondary metabolite pigments which include flavanols, anthocyanins, isoflavones and flavones, flavanones (Winkel-Shirley, 2002). Flavonoids represent the largest class of polyphenyols (>8000 metabolites) and share a three-carbon chain in which two aromatic rings are linked to form a specific class of flavonoid. A minimum of 54 flavonoid molecules have been identified in *Arabidopsis thaliana* of which 35 are flavonols,11 are anthocyanins and 8 are proanthocyanidins (Saito *et al*., 2013). Flavonols are known to absorb light between 280-320 nm and the flavonol concentration increases in leaf tissue under UV-B light (Agati *et al*., 2011).

The flavonoid biosynthesis pathway results from the combination of the phenylpropanoid and polyketide pathways (Satio *et al*., 2013). The first enzyme of the flavonoid biosynthesis pathway is CHS (TT4). CHS catalyses the reaction between p-coumaroyl CoA and malonyl-CoA to form a triketide which undergoes spontaneous cyclization to form naringenin chalcone. CHI catalyses naringenin chalcone to naringenin. F3H then catalyses naringenin to form dihydrokaempferol. Dihydrokaempferol is synthesised into the flavonol kaempferol through FLS or undergoes an additional step to form dihydroquercetin through F3'H (TT7). FLS can synthesise either dihydroquercetin or kaempferol into the flavonol quercetin (figure 12). These flavonols are involved in the absorbance of UV-B.

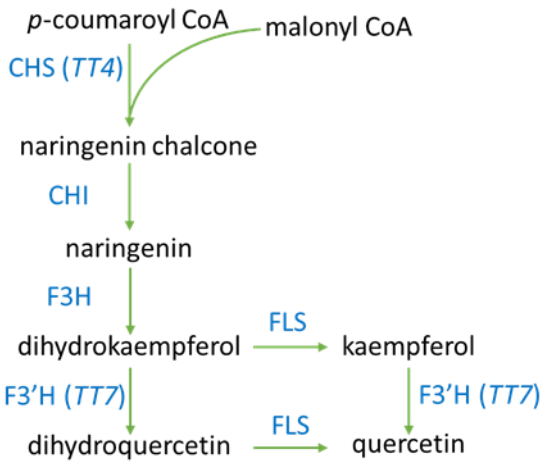

Figure 12. A diagrammatic section of the Arabidopsis flavonoid biosynthesis pathway. Enzymes are coloured blue, the genes for *CHS* and *F3'H* are included in brackets. Figure adapted from Saito *et al*. (2013).

Flavonoids are involved in many biological responses including plant hormone signalling, pollen tube germination, colouration in angiosperms and protecting plants from UV-B induced

damage (Li *et al*., 1993; Mol *et al*.,1998). The role of flavonoids in protecting plants against UV-B-induced damage was first demonstrated by Li *et al*. (1993), using the flavonoid biosynthesis mutants *tt4* (lacking chalcone synthase) and *tt5* (lacking chalcone isomerase). Both mutants were hypersensitive to UV-B induced damage. Flavonoids are sequestered in vacuoles within the epidermis of a plant to absorb UV-B, thereby acting as a sunscreen (Stracke *et al*., 2010). Flavonols absorb light between 280-320 nm making them ideal for absorbing potentially harmful levels of UV-B. As well as providing plants with an important defence against UV-B damage, flavonoids have been shown to enhance Arabidopsis cold acclimation in multiple accessions (Hannah *et al*., 2006). Korn *et al*. (2008) further demonstrated a strong correlation between flavonoid content and freezing tolerance following crosses between Arabidopsis accessions. The importance of flavonoids during low temperatures has also been demonstrated using flavonoid biosynthesis mutants. *tt4*, *tt5* and *tt6* (deficient in *CHS*, *CHI* and *FLS*, respectively) all showed a significant reduced survival when exposed to low temperatures when compared to WT plants. (Schulz *et al*., 2015).

In addition to absorbing UV-B, phenolics have been suggested to be involved in thickening the cell wall and preventing ice nucleation within the cell (Chalker-Scott, 1992). Membrane stabilization has also been proposed as a role of flavonoids during freezing temperatures (Schulz *et al*., 2016). Therefore, UV-B-mediated induction of flavonoids may enhance cold acclimation and whole plant freezing tolerance. In this chapter, the role of UV-B in plant cold acclimation was investigated in Arabidopsis using whole plant freezing tolerance assays, qPCR of flavonoid biosynthesis gene transcripts and thin layer chromatography (TLC) of flavonoids.

4.2 Results

All genotypes (WT, *tt4*, *tt7*) were grown at 20 °C for 2 weeks with and without supplementary UV-B (1 µmol $m^{-2}s^{-1}$). Half of each treatment group were subsequently cold acclimated under continuous red and blue light for 24 h, with and without UV-B supplementation (treatment groups: UV-B CA, UV-B non-CA, no-UV-B CA and no-UV-B non-CA). For qPCR transcript abundance analysis plant aerial tissue was harvested after cold acclimation. For whole plant freezing tolerance assays after the cold acclimation phase all plants were subjected to a -6 °C freezing treatment for 24 h and a 2-week recovery period.

4.2.1 UV-B and cold reduce survival in tt4 plants

Percentage survivability was measured in WT and *tt4* plants using a whole plant freezing tolerance assay. To investigate the effect of temperature, UV-B and genotype on freezing tolerance, a 3-way ANOVA was performed. This analysis revealed a statistically significant effect on survivability with temperature treatment [F(1,23) = 1656.818, P <0.001]; UV-B treatment [F(1,23) = 20.455, P <0.001]; and genotype [F(1,23) = 596.455, P <0.001]. Statistically significant interactions between variables were also identified between, temperature and UV-B [F(1,23) = 32.818, P <0.001]; UV-B and genotype [F(1,23) = 11, P = 0.004]; temperature and genotype [F(1,23) = 720.091, P <0.001]; genotype, temperature and UV-B [F(1,23) = 20.455, P <0.001]. A Tukey post hoc analysis was performed to analyse which treatments were statistically significant from one another. All non-cold-acclimated plants showed significantly reduced survivability compared to the cold-acclimated plants (P <0.001) except cold-acclimated *tt4* plants treated with UV-B which was not significantly different from non-cold-acclimated *tt4* treated with UV-B. UV-B did not significantly effect survivability in non-cold acclimated WT and *tt4*, or in cold-acclimated WT plants. However, UV-B treatment significantly reduced survivability in cold acclimated *tt4* plants (P <0.001; n = 60; figure 13 and 15). Together, these data suggest that cold acclimation enhances freezing tolerance in the experimental conditions used here. UV-B supplementation had no effect on freezing tolerance in WT plants and decreased freezing tolerance in *tt4* mutants. The results further show that *CHS* expression is required for effective cold acclimation, supporting the importance of flavonoids in this process.

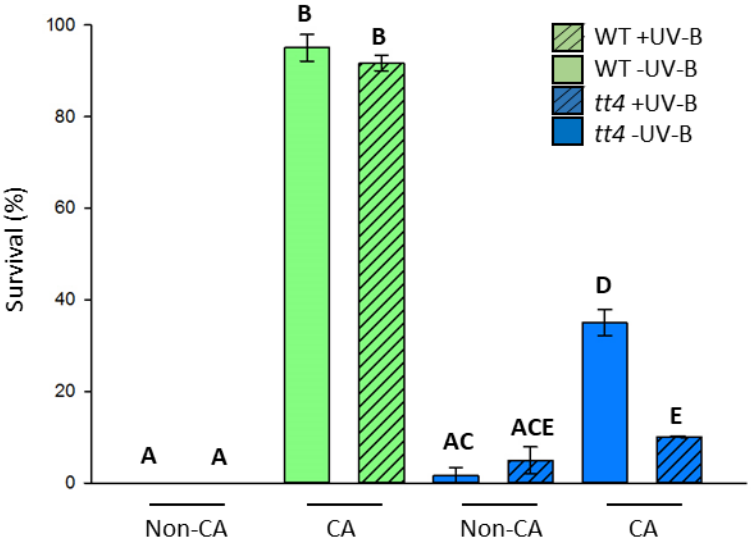

Figure 13. Percentage survival of 2-week-old WT and *tt4* seedlings treated with cold and UV-B after a 24 h freezing treatment at -6 °C. Plants were grown for 2 weeks with and without supplementary UV-B (1 µmol $m^{-2}s^{-1}$). Half of all plants were cold acclimated for 24 h at 4 °C. Following freezing treatment, a 2-week regrowth phase was allowed before calculating percentage survival. Different letters indicate statistically significant differences between treatments (P <0.05). Non-CA = non-cold acclimated, CA = cold acclimated. Striped columns indicate exposure to UV-B. Green columns = WT. Blue columns = tt4. n = 60, SE bars.

4.2.2 UV-B and cold reduce survival in tt7 plants

Percentage survivability was also measured in *tt7* plants using a whole plant freezing tolerance assay. A 3-way-ANOVA revealed that there was a statistically significant effect on survivability with temperature treatment [F(1,23) = 2102.5, P <0.001]; UV-B treatment [F(1,23) = 28.9, P <0.001]; and genotype [F(1,23) = 624.1, P <0.001]. Statistically significant interactions between variables were also identified, temperature and UV-B [F(1,23) = 28.9, P <0.001]; UV-B and genotype [F(1,23) = 16.9, P = 0.001]; temperature and genotype [F(1,23) = 624.1, P <0.001]; genotype, temperature and UV-B [F(1,23) = 16.9, P = 0.001]. A Tukey post hoc analysis was performed to analyse which treatments were statistically significant from one another. 0% survival was recorded in the non-cold-acclimated WT and *tt7* plants. Cold-acclimated WT plants treated with UV-B showed a significantly higher survival rate than cold-acclimated *tt7* plants in the same conditions (P <0.001). Cold-acclimated WT plants grown without UV-B had a significantly higher survival rate than the cold acclimated *tt7* plants grown in the same conditions (P <0.001). UV-B had no discernible effect on survivability in WT plants. However, UV-B treatment decreased survivability in cold-acclimated *tt7* plants (P <0.001; n = 60; figures 14 and 15). Together, these data support current literature that cold acclimation enhances freezing tolerance in the experimental conditions used. They further show that F3'H expression is required for effective cold acclimation, suggesting the importance of flavonoids in this process. However, UV-B supplementation had no effect on freezing tolerance in WT plants and decreased freezing tolerance in *tt7* mutants.

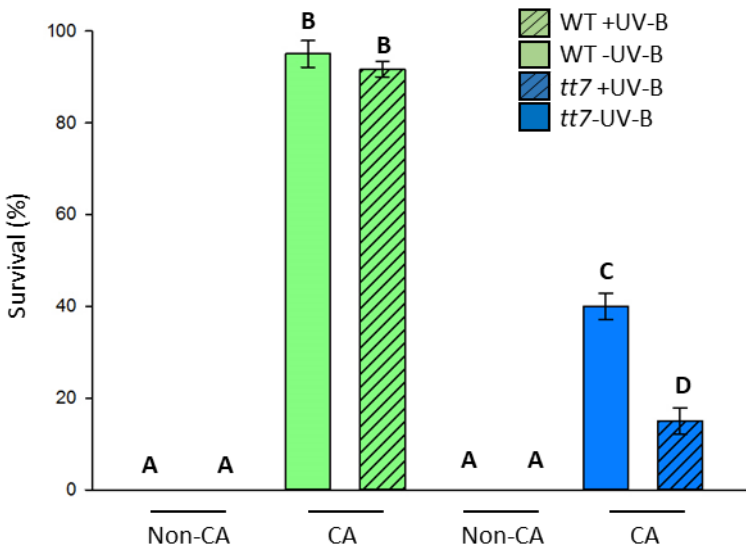

Figure 14. Percentage survival of 2-week-old WT and *tt7* seedlings treated with cold and UV-B after a 24 h -6 °C freezing treatment. 50% of all plants were exposed to UV-B (1 µmol $m^{-2}s^{-1}$) during the growth phase. Prior to freezing treatment 50% of all plants were cold acclimated for 24 h at 4 °C in the light ± UV-B. After the freezing treatment a 2-week regrowth phase was allowed before calculating percentage survival. Different letters indicate statistically significant differences between treatments (P <0.05). Non-CA = non-cold-acclimated, CA = cold-acclimated; striped columns indicate exposure to UV-B; green columns = WT, blue columns = *tt7*. n = 60, SE bars.

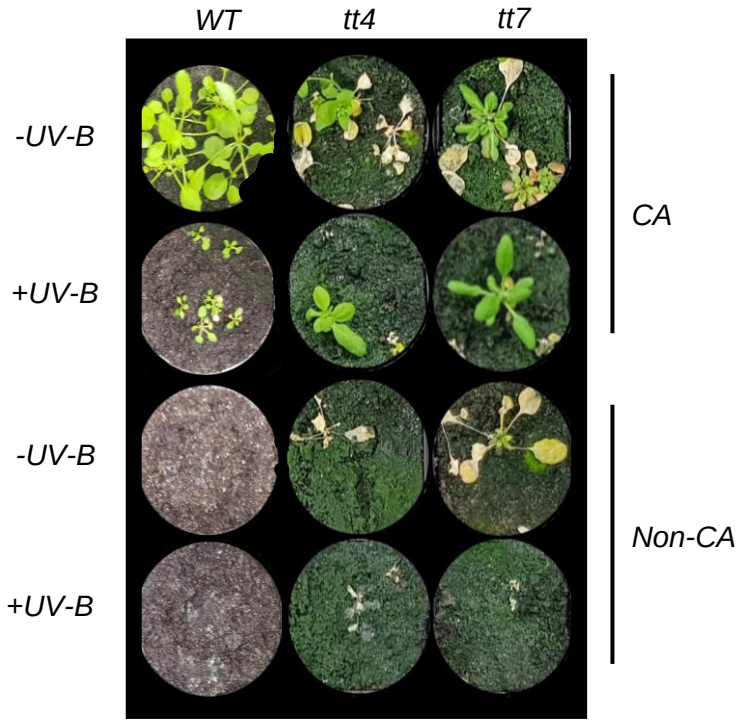

Figure 15. Photograph of cold- and UV-B-treated WT, *tt4* and *tt7* mutant plants, 2 weeks after being subjected to a -6 °C freezing treatment. Treatment conditions were cold-acclimated (CA), non-cold-acclimated (non-CA) with UV-B (+UV-B) and without UV-B (-UV-B).

4.2.3 Cold and UV-B may influence CHS transcript abundance

CHS transcript abundance was measured in WT and *uvr8-6* plants to assess the impact of UV-B and cold acclimation on flavonoid production. Plants that were cold acclimated showed higher *CHS* transcript abundance than non-cold-acclimated controls (figure 16). Cold-acclimated WT and *uvr8-6* plants that additionally received UV-B supplementation showed higher *CHS* transcript abundance than plants grown without UV-B. Cold acclimated *uvr8-6* plants grown without UV-B showed higher *CHS* transcript abundance than the corresponding WT controls. When treated with UV-B, however, this pattern was reversed. WT plants grown in the presence of UV-B, without cold acclimation, showed a 3-fold increase in *CHS* transcript abundance compared to No-UV-B controls. No UV-B-induced differences in *CHS* transcript abundance were observed in non-cold acclimated *uvr8-6* plants (figure 16). Despite visible trends being observed, a Kruskal-Wallis test determined that no statistically significant differences existed between any of the treatment groups.

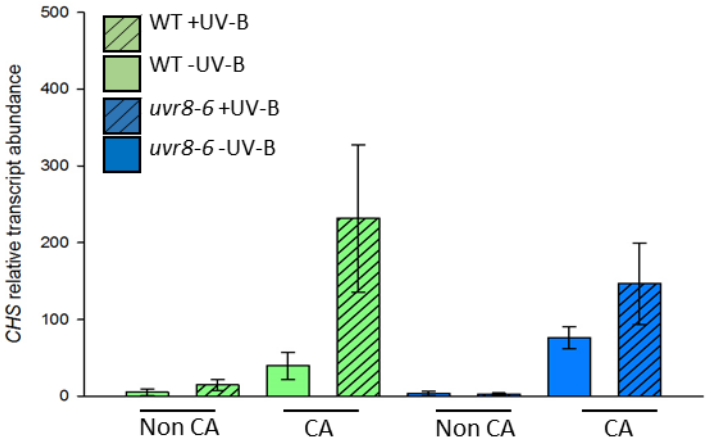

Figure 16. Relative transcript abundance of *CHS* in cold-treated WT and *uvr8-6* plants grown with and without UV-B (1 µmol $m^{-2}s^{-1}$). At 2 weeks, half the plants were cold acclimated in the light for 24 h at 4 °C (with and without UV-B) and half remained at 20 °C. Non-CA = non-cold acclimated, CA = cold-acclimated; striped columns indicate exposure to UV-B; green columns = WT, blue columns = *uvr8-6*. n = 3, SE bars.

4.2.4 Cold and UV-B may enhance flavonoid concentration in WT plants

Qualitative analysis of plant flavonoid content was performed on WT and *uvr8-6* genotypes using TLC. Higher intensity of the coloured bands of the silica plate corresponds to higher concentration of flavonoid pigments. Samples from WT plants showed greater colour intensity than *uvr8-6*, suggesting a lack of flavonoid production in *uvr8-6* mutants (figure 17). The supplementation of UV-B increased the intensity of both quercetin and kaempferol bands in WT plants, with and without cold acclimation (figure 17; quercetin = orange rectangle, kaempferol = green rectangle). The combination of cold and UV-B resulted in the highest band intensity, suggesting the highest accumulation of flavonoids.

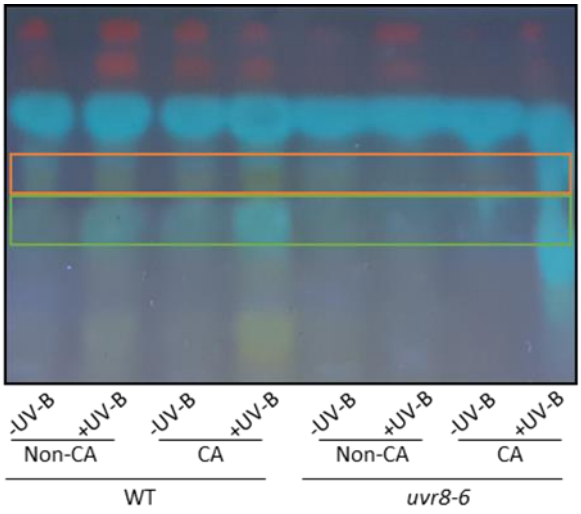

Figure 17. Flavonoid pigments in 2-week-old Arabidopsis leaves (WT and *uvr8-6*). Qualitative analysis of flavonoids was performed using TLC. Plants were grown with and without UV-B (1 µmol $m^{-2}s^{-1}$). Half the plants were cold acclimated in the light for 24 h at 4 °C with and without UV-B before tissue collection. The flavonoids quercetin and kaempferol are indicated by orange and green rectangles, respectively. The intensity of the colour bands indicates an increase in pigment levels. Non-CA = non-cold-acclimated, CA = cold-acclimated; - UV-B received no UV-B treatment, + UV-B received UV-B treatment.

4.3 Discussion

Whole plant freezing tolerance assays were performed using *tt4* and WT genotypes to assess the importance of CHS in protecting Arabidopsis against freezing damage. In both WT and *tt4* plants, cold acclimation significantly increased survival (figure 13). These observations support data showing that cold acclimation enhances Arabidopsis freezing tolerance (Guy *et al*., 1985). Cold-acclimated WT plants grown without UV-B showed a significantly higher percentage survival than *tt4* mutants grown in similar conditions. These data suggest that flavonoid synthesis enhances plant freezing tolerance and support the findings of Schulz *et al*. (2015) who showed that all flavonoids were upregulated after cold acclimation. The supplementation of UV-B had no significant effect on survivability in the cold acclimated WT plants but interestingly significantly decreased survivability in cold-acclimated *tt4* plants. These observations support the involvement of CHS in protecting plants against UV-B damage (Li *et al*.; 1993; Koostra, 1994). Together these findings support published data suggesting the CHS enzyme is central to both cold and UV-B protection.

A freezing tolerance assay for *tt7* mutants was also performed. Cold acclimated plants showed significantly increased survival compared to non-cold acclimated plants, however cold acclimated *tt7* plants had significantly reduced survivability compared to the WT control. These data suggest that for freezing protection, plants are dependent upon the flavonoid quercetin. The *tt7* mutant lacks the key enzyme F3'H (flavonoid 3-hydroxylase) which catalyses the synthesis of dihydrokaempferol into dihydroquercetin which can then be synthesised to quercetin by FLS (flavonol synthase), FLS can also synthesise kaempferol into quercetin (Mehrtens *et al*., 2005) (figure 12). Data from both the *tt4* and *tt7* freezing tolerance assays suggest a strong involvement of flavonoids in protecting plants not only from freezing conditions but also from UV-B. The effectiveness of cold acclimation in promoting plant survival following freezing treatment prevented the analysis of whether UV-B could enhance this process. High levels of survival in cold-acclimated plants (approaching 100%, except in the UV-B-treated and cold acclimated *uvr8-6*) and 0% survival in non-cold-acclimated plants prevented analysis of any effect of UV-B in addition to cold. Further experiments using a more severe freezing treatment, a reduced cold acclimation period or a more sensitive assay, such as electrolyte leakage, may elucidate the effect of UV-B on freezing tolerance. The electrolyte leakage assay would be a primary candidate as it measures damage to the plant through the leakage of ions and has been successfully used to measure damage induced by low temperatures (Xing *et al*., 2011; Hemsley *et al*., 2014). This may be a more accurate way of assessing the impact of UV-B in protecting plants against low temperature damage.

qPCR was used to assess if cold acclimation and UV-B could additively upregulate *CHS* transcript abundance (figure 16). A Kruskal-Wallis test showed no statistically significant interactions. This was likely due to a small sample size (n = 3) in combination with variation in the data. Despite these issues, some trends could be identified. *CHS* transcript abundance was increased in WT and *uvr8-6* by cold acclimation suggesting that *CHS* is upregulated during low temperatures. This complements studies showing flavonoid production during low temperatures (Schulz *et al*., 2015). A larger increase in *CHS* transcript abundance was observed when cold acclimated WT and *uvr8-6* plants received supplementary UV-B treatment. UV-B is well documented to upregulate *CHS* (Mehrtens *et al*., 2005). The data presented here may suggest that cold acclimation and UV-B act synergistically to upregulate *CHS*. Analysis of the TLC plate appears to show there is accumulation of the flavonoids quercetin and kaempferol in WT plants when receiving cold acclimation and UV-B. This supports the qPCR data trend that *CHS* is further upregulated when WT plants are cold acclimated and treated with UV-B. However, the TLC plate shows comparatively little to no upregulation of quercetin or kaempferol in *uvr8-6* plants. This concurs with the data trends of the qPCR which showed severely reduced expression of *CHS* compared with WT plants.

Together, the data presented in this chapter suggest that UV-B enhances the production of flavonoids during cold acclimation and that this occurs, in part, via synergistic up-regulation of *CHS* expression by cold and UV-B treatments. This is further supported by the TLC data which suggest that the flavonoids kaempferol and quercetin accumulate during cold and UV-B in WT plants. Whole plant survival assays confirmed the importance of flavonoid biosynthesis for freezing tolerance but were not sensitive enough to determine whether growth in UV-B can enhance WT cold acclimation.

CHAPTER 5: DISCUSSION

5.1 UV-B enhances cold-induced accumulation of *COR* gene transcripts, but not whole plant survival following freezing

UV-B supplementation has been demonstrated to guard against freezing-induced damage in multiple species. Dunning *et al*. (1994) found that phenolic leakage (a measure of freezing damage) was reduced in UV-B supplemented Rhododendron plants. Yang *et al*. (2007) additionally showed that *Triticum aestivum* produced more ROS scavenging enzymes under UV-B supplementation, providing plants with enhanced protection against freezing temperatures. These findings suggest crosstalk between the low temperature and UV-B signalling pathways. Crosstalk is a phenomenon in plants where multiple signalling pathways converge and has been well investigated in the signalling pathways of cold, drought and salt stress. Since all these stressors cause dehydration, each signalling pathway is involved in protecting the plant against dehydration regardless of which stress the plant is subjected to (Seki *et al*., 2003; Dai *et al*., 2007). Crosstalk between light and cold has not, however, been as thoroughly investigated. The integration of light and temperature signals is very important in the cold acclimation process and it has been demonstrated to be mediated, in part, through phytochrome photoreceptors (Olsen *et al*., 1997; Franklin and Whitelam, 2007). Catalá *et al*., (2011) have demonstrated that the bZIP transcription factor HY5 is important for integrating light and low temperature signals to promote full cold acclimation. HY5 is a key regulator of plant photomorphogenesis and is repressed by COP1 in the dark (Chen and Xiong, 2008). In the presence of light, phytochromes and cryptochromes negatively regulate COP1. UV-B activates UVR8, which monomerises and binds to COP1, stabilising HY5 and promoting the expression of downstream genes (Heijde and Ulm, 2012) (figure 6). HY5 is required for full cold acclimation and is responsible for the expression of at least 10% of all cold-inducible genes (Catalá *et al*., 2011). This is achieved by HY5 binding to an LTRE in the Z-box which allows cold inducible genes such as *CAB1* to be expressed (Catalá *et al*., 2011). Knowing the importance of light signals in the cold acclimation process, UV-B was investigated to understand if UV-B could enhance freezing tolerance by enhancing cold acclimation. This was examined by measuring the transcript abundance of two *COR* genes, *COR15a* and *COR47*.

Analyses of transcript abundance showed that when WT plants were treated with a combination of cold and UV-B, *COR15a* and *COR47* relative transcript abundance increased (figures 8 and 9). This indicates crosstalk between the signalling pathways of UV-B and cold acclimation. Although statistical analysis of the results from this study did not reveal any significant differences between treatment groups, clear trends in the data warrant further investigation. Time prevented further replication. To confirm *COR15a* and *COR47* trends in

WT plants, more replicates would be required. Further replication may also reveal a trend in the *uvr8-6* plants and therefore elude to whether UVR8 activity is required for UV-B-enhanced expression of *COR15a* and *COR47*.

Whole plant freezing tolerance assays were performed to assess if UV-B could directly increase plant survival following freezing temperatures. In contrast with Dunning *et al*., (1994), the freezing tolerance assays conducted here using WT and *uvr8-6* plants did not show UV-B to enhance freezing tolerance. This was due to high levels of survival (approaching 100%) in the cold acclimated plants (except in the UV-B-treated *uvr8-6* mutants) and 0% survival in the non-cold acclimated plants (figure 10). It is possible that the assay used was not sensitive enough to detect any impact that UV-B may have had on survivability as previously mentioned in chapter 3. However, it is also possible that a different outcome compared with Dunning *et al*., (1994) was achieved due to measuring whole plant survival instead of leaf disc damage. Whole plant analysis was chosen over leaf discs as it is more representative of how plants cope under these stresses in a natural environment. Alternatively, Arabidopsis may respond differently to cold and UV-B than Rhododendron and Wheat. The *uvr8-6* mutant was used to help elucidate whether any effect UV-B was having in increasing cold tolerance was acting through the UVR8 photoreceptor. Due to high levels of survival in cold acclimated plants and low levels of survival in the non-cold acclimated plants, it was not possible to ascertain whether UV-B impacted survivability and therefore UVR8's involvement could not be gauged. This mutant does not appear to be appropriate for the freezing tolerance assay as *uvr8-6* plants exposed to UV-B and freezing temperatures appear to receive a double stress which reduced survivability (figure 10). As *uvr8-6* plants lack the UVR8 photoreceptor, they lack the means to detect UV-B and activate the flavonoid biosynthesis pathway. Flavonoids absorb UV-B wavelengths preventing ROS formation, protecting the plant (Kootstra, 1994). The application of UV-B and a -6 °C freezing treatment may therefore have resulted in considerable tissue damage in *uvr8-6* mutants. In future work, whole plant freezing tolerance assay experiments could be replaced by electrolyte leakage assays. This would be more informative as damage can be measured through the leakage of ions instead of the binary results (dead or alive) collected from the whole plant freezing survival assays. This method has been successfully used to measure damage induced by low temperatures (Uemura *et al*., 2006; Hemsley *et al*., 2014) and would allow investigation of plant responses to multiple temperatures.

Discrepancies between transcript abundance and freezing survival assays may be explained if transcripts are prevented from being translated into proteins. To analyse this a quantitative western blot could be used to ensure that *COR* transcripts are translated into protein. The electrolyte leakage assay could also be used to investigate the effects of UV-B supplementation on cold acclimation in *hy5* and *hyh* mutants. HY5 is known to be involved in

regulating the expression of flavonoids through UV-B (Roman *et al*., 2004). It would therefore be interesting to assess if HY5 is involved in the integration of UV-B and low temperature signalling since it has already been linked with phytochrome signalling and low temperature (Catalá *et al*., 2011).

5.2 UV-B may conditionally enhance freezing tolerance via the up-regulation of flavonoids

Flavonoids were investigated in this study as they are known to be involved in protecting plants from UV-B damage (Li *et al*., 1993; Stracke *et al*., 2010) and freezing temperatures (Hannah *et al*., 2006; Korn *et al*., 2008). This makes the flavonoid biosynthesis pathway a point of potential crosstalk between UV-B and low temperature signalling. To assess this, whole plant freezing tolerance assays were performed using the flavonoid mutants *tt4* and *tt7*. The *TT4* gene encodes chalcone synthase, the first enzyme in the flavonoid biosynthesis pathway and *TT7* encodes F3'H which is a crucial enzyme in the synthesis of quercetin further downstream (figure 12). The impaired freezing tolerance displayed by *tt4* plants suggests that CHS plays an important role in cold acclimation and freezing tolerance in Arabidopsis (figure 13). Analysis of *CHS* transcript abundance showed increased *CHS* accumulation when plants received both a cold acclimation treatment and UV-B treatment, although results were not statistically significant (figure 16). These results may suggest that UV-B and cold act synergistically to enhance *CHS* abundance although more replicates would need to be performed to confirm this. F3'H mutants (*tt7*) were tested using whole plant freezing tolerance assays to identify if kaempferol or quercetin were important in protecting plants from freezing temperatures and UV-B (figure 14). In contrast to the results of Shulz *et al*. (2016), *tt7* mutants showed similar levels of survival after freezing treatment to *tt4* plants. They additionally showed significantly lower levels of survival than WT plants when cold acclimated and treated with UV-B. *tt4* and *tt7* mutants are defective in quercetin biosynthesis. Together, these results suggest that quercetin is an important component in both UV-B protection and cold acclimation and may be involved in integrating the two signalling pathways.

To attempt to quantify flavonoid abundance in cold- and UV-B-treated plants, a Dualex spectrometer was used to measure epidermal pigments (Goulas *et al*., 2004). Measurements of chlorophyll accumulation were also taken in parallel to assess the potential impact of cold and UV-B on photosynthesis. This allowed multiple measurements to be taken without damaging the plant tissue which could potentially change the concentration of phenolic compounds. The small leaf size of plants grown in UV-B, did however, prevent reliable measurements. Plants were grown for an additional 2 weeks (4 weeks total) to increase leaf

size area but this failed to resolve the issue, so the experiment was discontinued due to time constraints (Appendix figure S8, S9 and S10). Adjusting light quality is an option that may allow greater leaf size and therefore the Dualex spectrophotometer to be used (Casal *et al*., 1987). This would allow chlorophyll and anthocyanin measurements to be taken in addition to flavonoids. To address the failed quantitative analysis of flavonoids, a quantitative chromogenic assay could be used to enable measurement from plants with small leaves (Julkunen-Tiitto *et al*., 2015). A qualitative TLC assay was subsequently used to gain an understanding of flavonoid accumulation (figure 17). The TLC results support *tt7* whole plant freezing tolerance assay data as coloured bands representing quercetin have a higher intensity in plants treated with cold acclimation and UV-B (figure 17). Levels of kaempferol and quercetin were low in *uvr8-6* plants suggesting that UV-B-induced flavonoid accumulation requires functional UVR8 protein. Collectively, these experiments highlight the importance of flavonoids in protecting plants from low temperatures and UV-B.

Another consideration for future work would be to manipulate the UV-B treatments administered to plants. Plants acclimate to UV-B over time, reducing differences in transcript abundance between treated and untreated plants (Jansen *et al*., 2010). A transient UV-B treatment may therefore act to enhance the expression of genes involved in UV-B and cold signalling. To optimise a transient UV-B treatment the time of day UV-B treatment is administered should be considered. UV-B responses are gated by the circadian clock and Arabidopsis UV-B induced expression of *CHS* and *HYH* have been shown to increase during approximately midday (Fehér *et al*., 2011; Horak and Farré, 2015). However, the highest *CBF* transcript abundance (an important determinant in cold acclimation) is found 4 h after dawn (Fowler *et al*., 2005). Therefore, the most effective time to treat plants with UV-B may lie at some point between dawn and midday.

5.3 Conclusions

Analyses of *COR15a* and *COR47* transcript abundance suggest that UV-B can enhance the cold acclimation process, although whole plant freezing tolerance assays did not support this conclusion. Analyses of *CHS* transcript abundance, whole plant freezing tolerance assays using flavonoid biosynthesis mutants (*tt4* and *tt7*) and TLC analysis all suggest that UV-B may enhance cold acclimation by increasing flavonoids. It is likely that a point of crosstalk between low temperature and UV-B signalling pathways exists at an early point in the flavonoid biosynthesis pathway. Despite observed crosstalk between UV-B and low temperature in the regulation of both *COR* genes and flavonoid synthesis, no evidence exists that suggests the *CBF* regulon regulates the flavonoid biosynthesis pathway or vice versa. This would mean UV-B acts on the flavonoid biosynthesis pathway and *CBF* pathway separately. The point at which UV-B is acting on the *CBF* pathway to enhance *COR* gene expression remains unknown. However, phytochrome and the circadian clock are known to be crucial for regulating freezing tolerance through *CBF*s. It is therefore possible that UV-B is influencing the expression *COR* genes through the *CBF* regulon.

Understanding how plants respond to environmental stresses is very important to agriculture. Many greenhouses utilise materials which block UV-B wavelengths. Work from Wargent *et al*., (2011) has shown that UV-B treatment of seedlings increased harvestable yield and photosynthetic output in field-grown *Lactuca sativa* (lettuce). These data suggest that UV-B treatment prior to planting may enhance the robustness of field-grown crops. Increased crop losses due to low temperatures aggravate problems of food security due to an ever-increasing world population and opportunities to enhance resistance to environmental stress should be maximised. The data in this thesis suggest that UV-B may enhance cold acclimation and therefore freezing tolerance and warrants further investigation into crosstalk between UV-B and low temperature signalling.

CHAPTER 6: APPENDIX

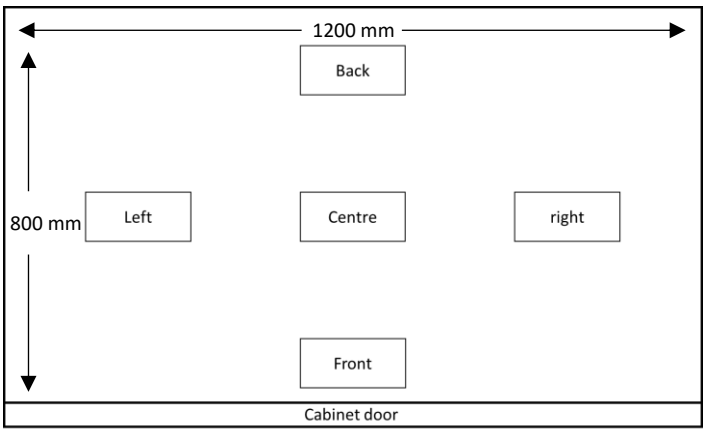

Figure S1. Schematic representation of growth cabinet showing where light measurements were taken (centre, left, right, back, front) and indicating growth cabinet shelf area.

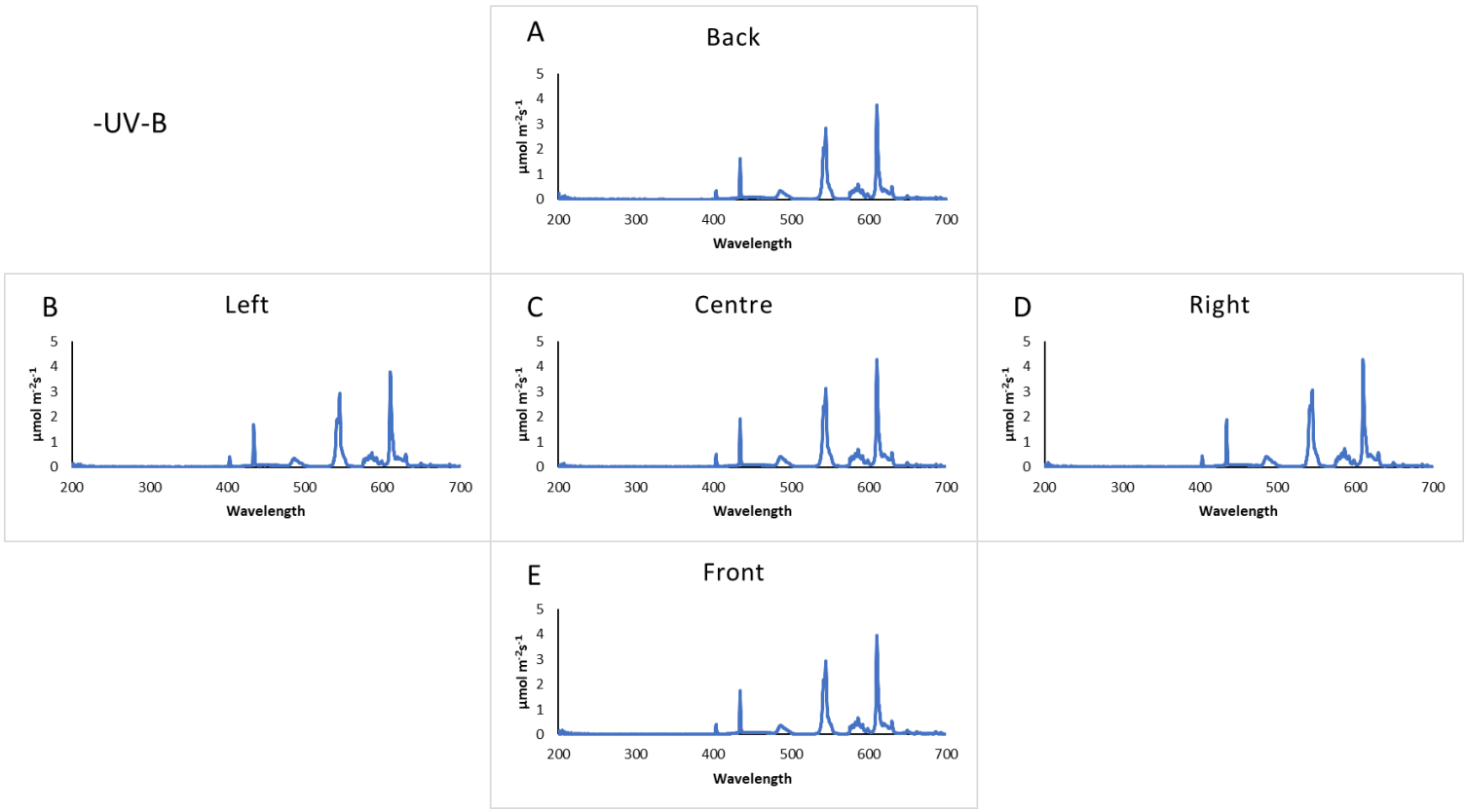

Figure S2. Light spectra from plant growth cabinet without supplementary UV-B. Measurement taken from: **A**) back of cabinet **B**) left of cabinet **C**) centre of cabinet **D**) right of cabinet **E**) front of cabinet. White light (100 µmol $m^{-2}s^{-1}$).

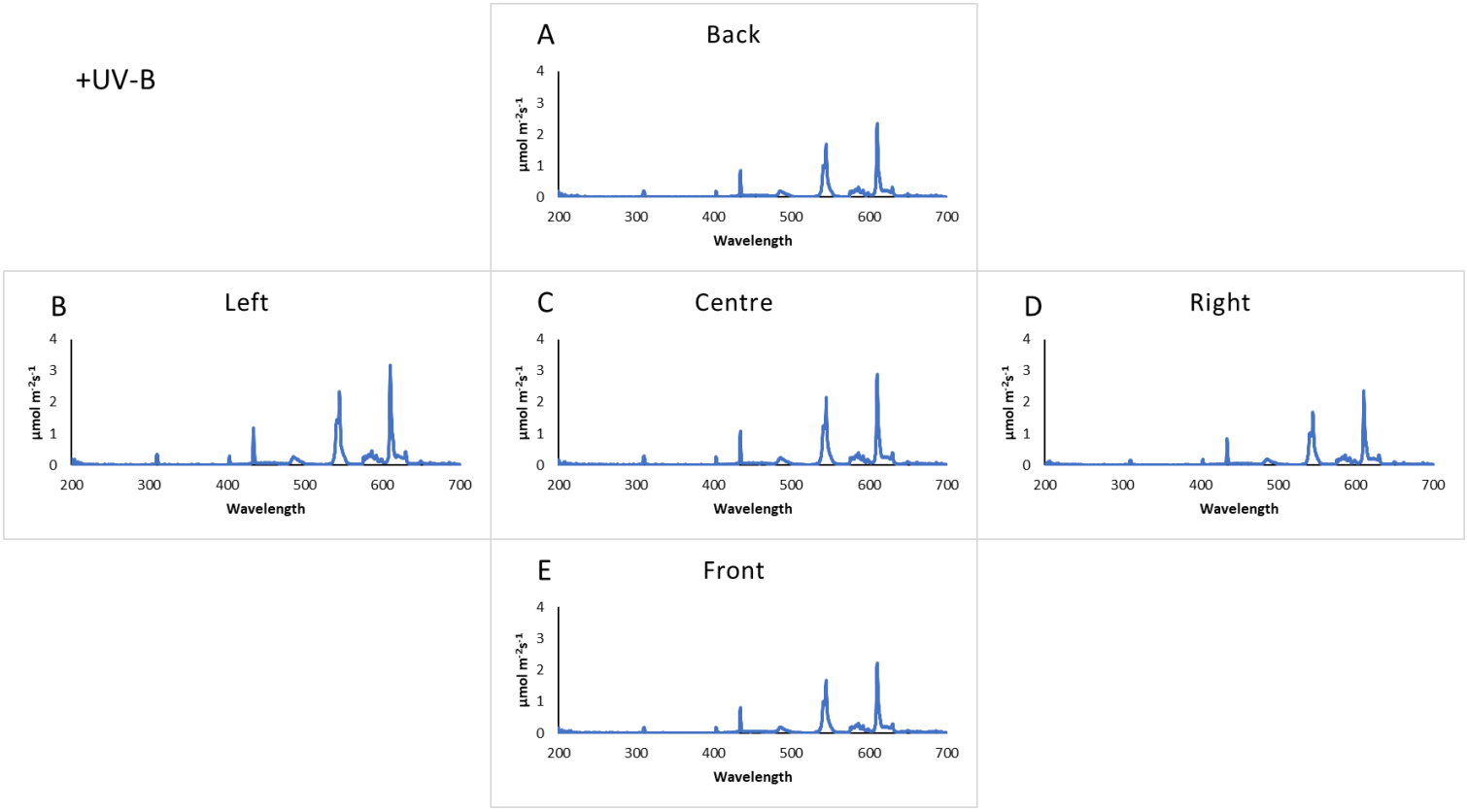

Figure S3. Light spectra from plant growth cabinet with supplementary UV-B. Measurement taken from: **A**) back of cabinet **B**) left of cabinet **C**) centre of cabinet **D**) right of cabinet **E**) front of cabinet. White light (100 µmol $m^{-2}s^{-1}$) UV-B (1 µmol $m^{-2}s^{-1}$).

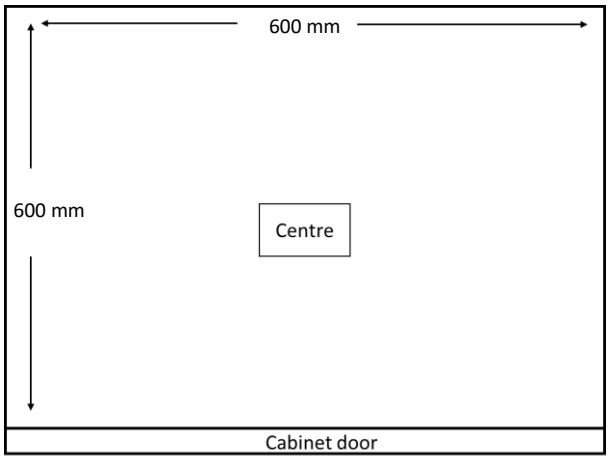

Figure S4. Schematic representation of the cabinet used for cold acclimation and plant freezing tolerance assays. Centre indicates where light measurements were taken from within the cabinet. Cabinet shelf area also indicated.

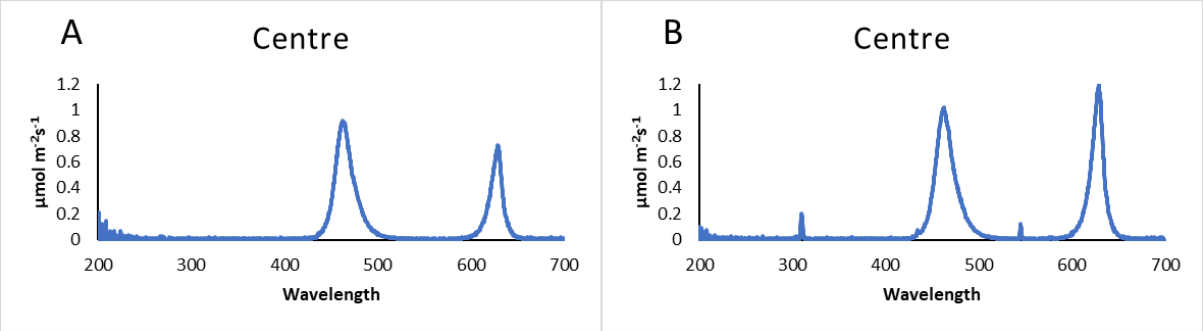

Figure S5. Light spectra from plant freezing cabinet **A**) without supplementary UV-B **B**) with supplementary UV-B. White light (70 µmol $m^{-2}s^{-1}$) UV-B (1 µmol $m^{-2}s^{-1}$).

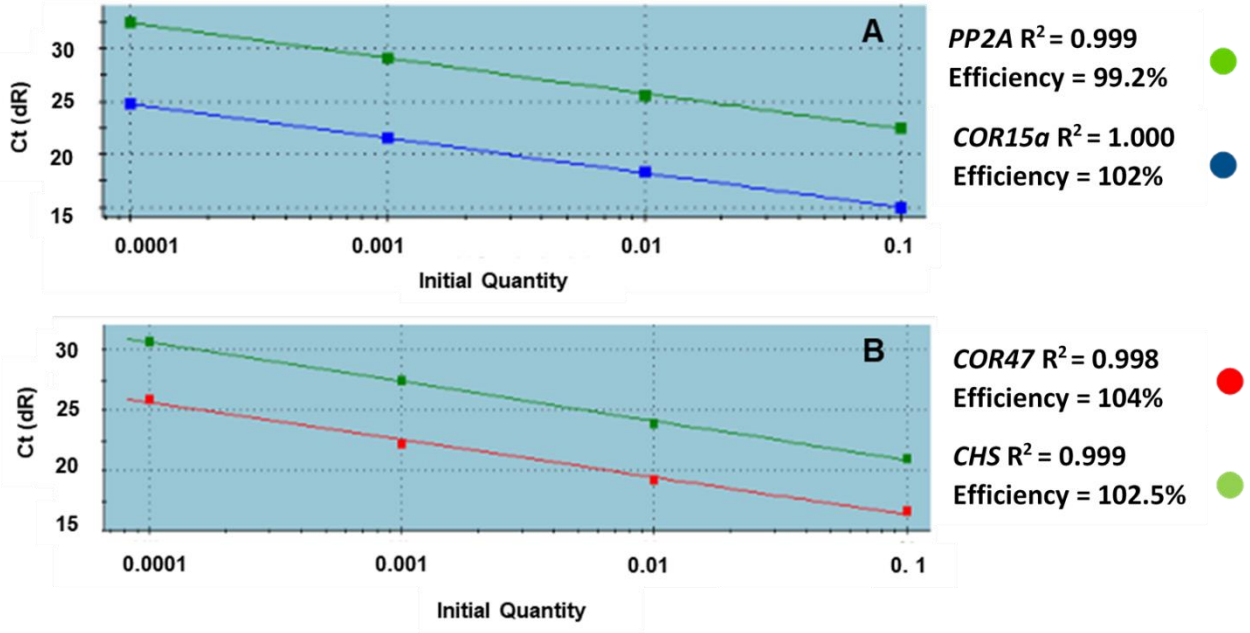

Figure S6. Primer efficiency standard curve from MXPro software. Initial quantity shows the dilution of the starting quantity of primer on the x axis. The Y axis displays the critical threshold (Ct). The efficiency and R^2 values are displayed.

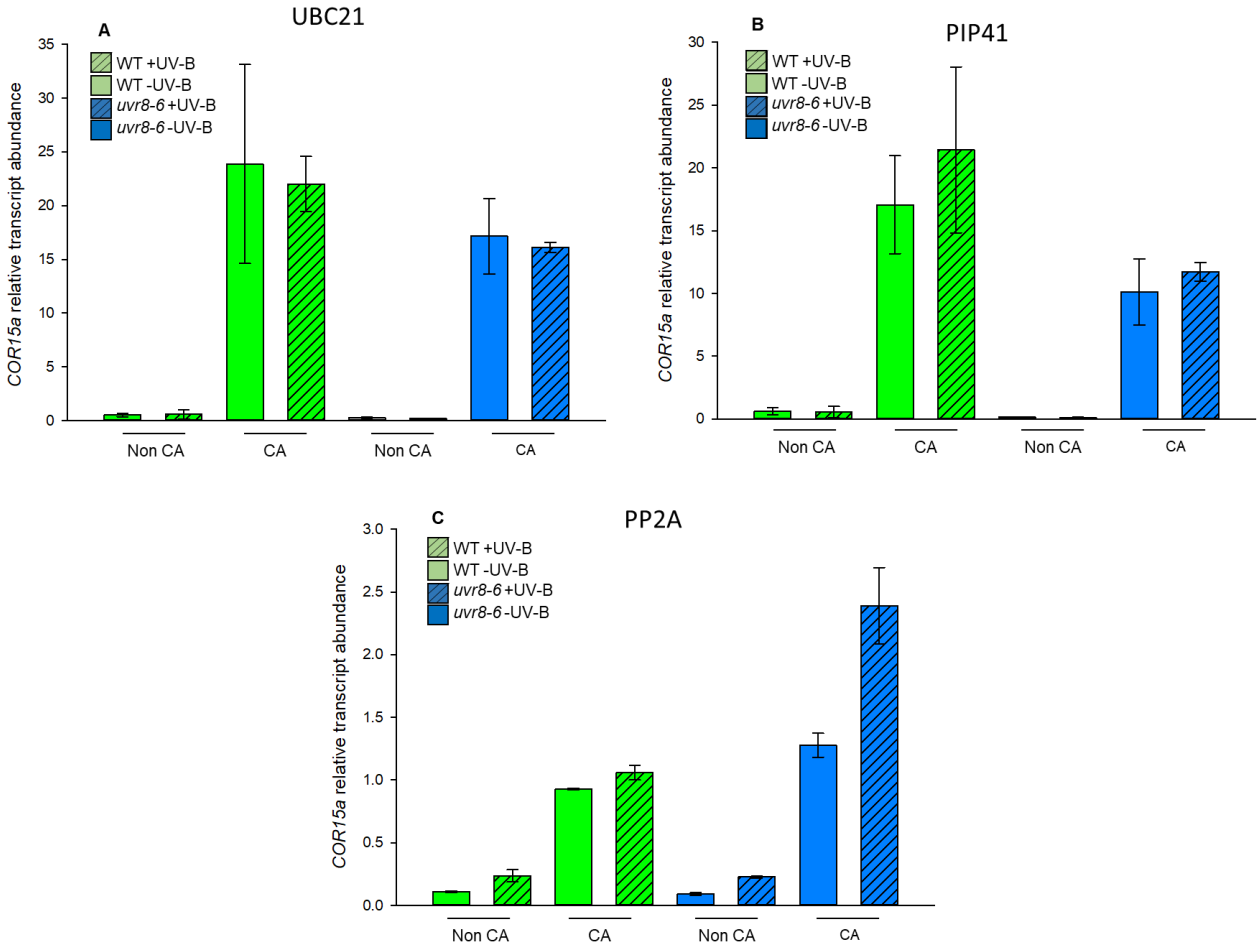

Figure S7. Testing of control genes **A**) *UBC21*, **B**) *PIP41* and **C**) *PP2A*, using relative transcript abundance of *COR15a* in 2-week-old Arabidopsis seedlings. 50% of all plants were exposed to UV-B (1 µmol $m^{-2}s^{-1}$) during growth phase. Before leaf tissue was collected for RNA extraction 50% of plants in each treatment were cold acclimated for 24 h at 4 °C. Non-CA = non-cold-acclimated, CA = cold-acclimated; striped columns indicate exposure to UV-B; green columns = WT, blue columns = *uvr8-6*. n = 3.

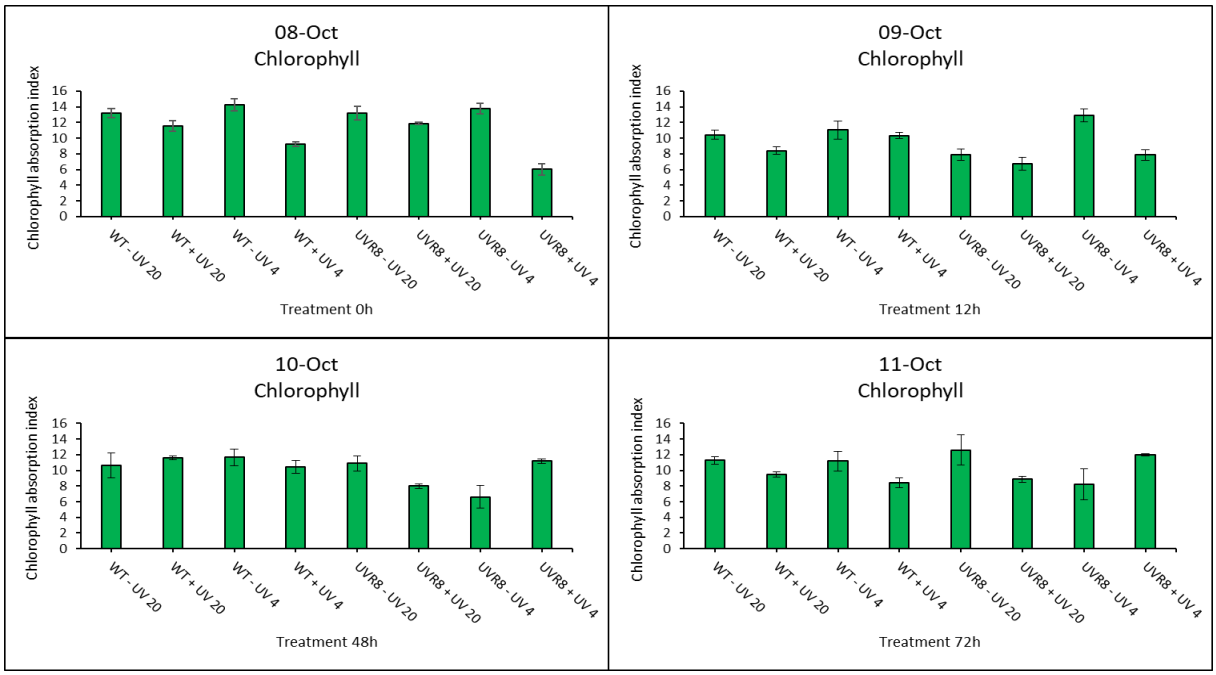

Figure S8. Chlorophyll measurement data collected using using a Dualex spectrometer. Measurements taken from the first leaf of the first rosette from time 0 h, 24 h, 48 h and 72 h. WT and *uvr8-6* plants were grown with and without UV-B (1 µmol $m^{-2}s^{-1}$) (indicated by -UV-B and UV-B respectively). Half the plants were cold acclimated in the light for 24 h at 4 °C with and without UV-B. Plants cold acclimated are indicated in the x axis with '4' and plants grown at 20 °C without cold acclimation are indicated by '20'.

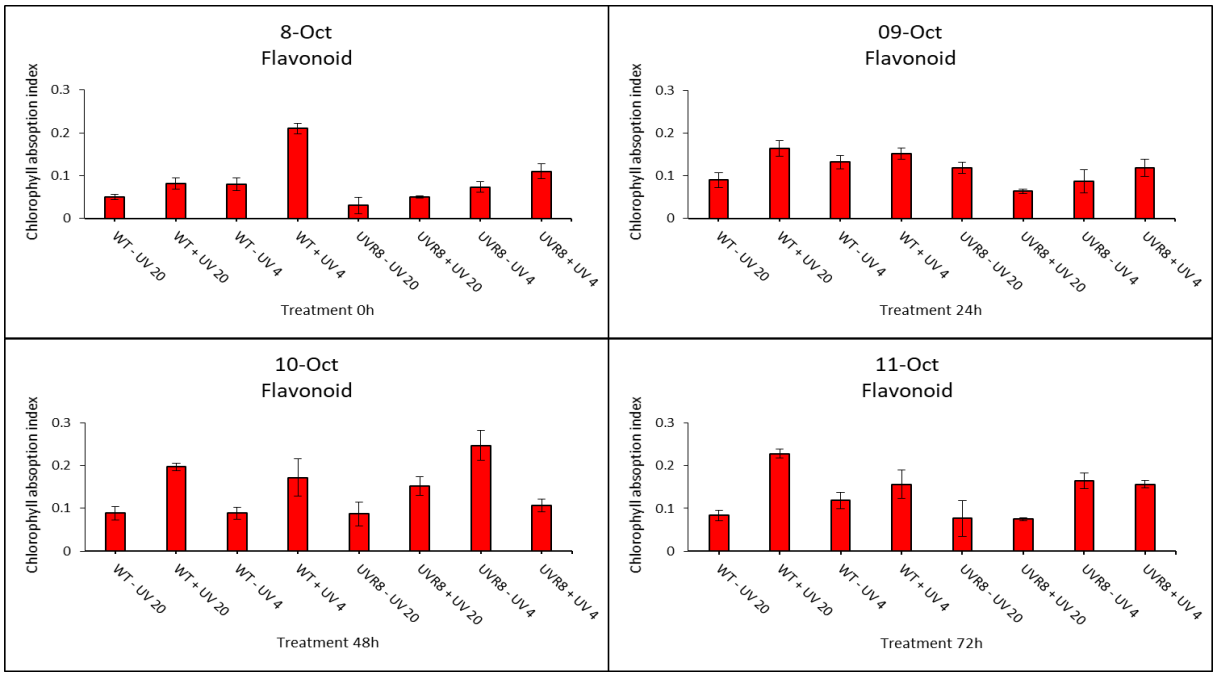

Figure S9. Flavonoid measurement data collected using a Dualex spectrometer. Measurements taken from the first leaf of the first rosette from time 0 h, 24 h, 48 h and 72 h. WT and *uvr8-6* Plants were grown with and without UV-B (1 µmol $m^{-2}s^{-1}$) (indicated by -UV-B and UV-B respectively). Half the plants were cold acclimated in the light for 24 h at 4 °C with and without UV-B. Plants cold acclimated are indicated in the x axis with '4' and plants grown at 20 °C without cold acclimation are indicated by '20'.

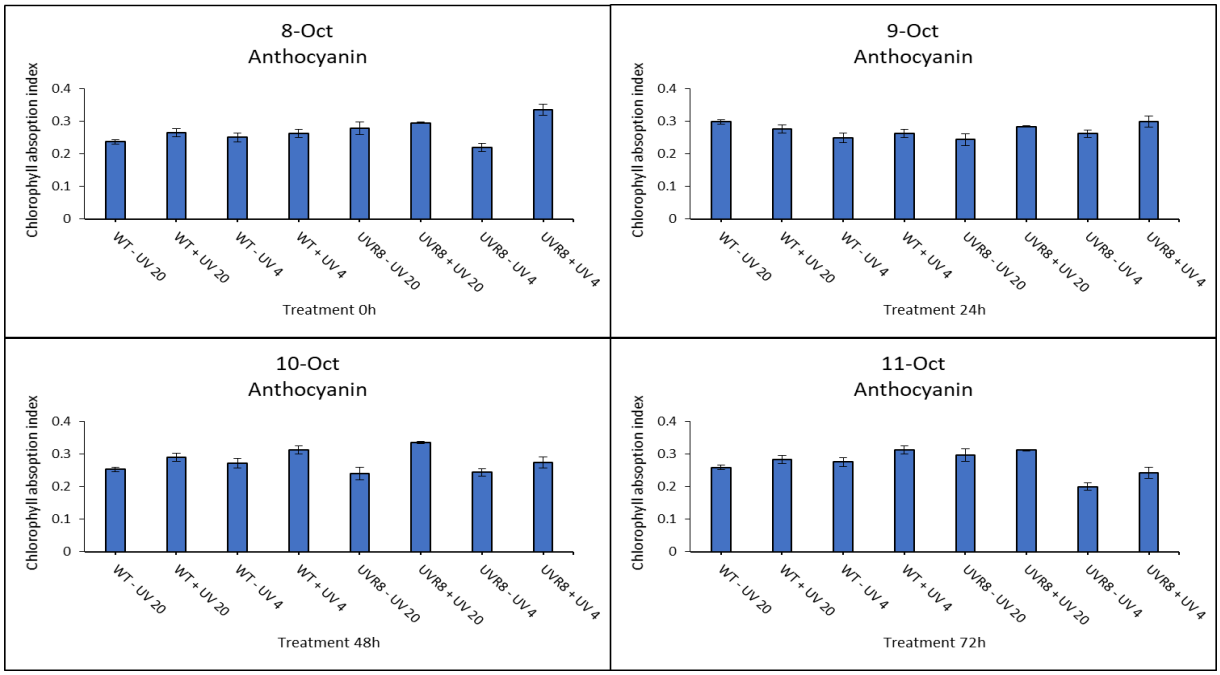

Figure S10. Anthocyanin measurement data collected using a Dualex spectrometer. Measurements taken from the first leaf of the first rosette from time 0 h, 24 h, 48 h and 72 h. WT and *uvr8-6* Plants were grown with and without UV-B (1 µmol $m^{-2}s^{-1}$) (indicated by -UV-B and UV-B respectively). Half the plants were cold acclimated in the light for 24 h at 4 °C with and without UV-B. Plants cold acclimated are indicated in the x axis with '4' and plants grown at 20 °C without cold acclimation are indicated by '20'.

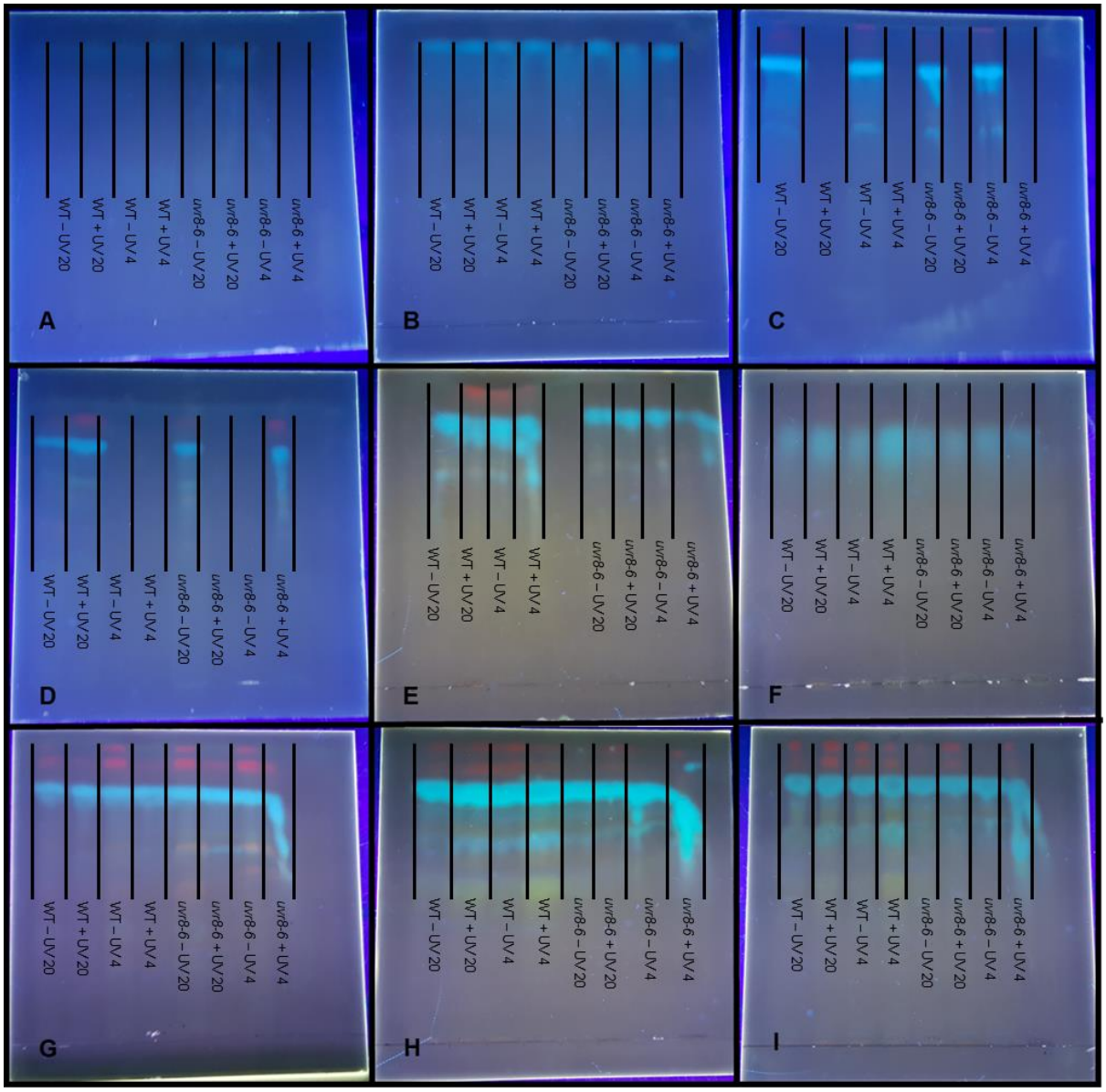

Figure S11. Flavonoid pigments in 2-week-old Arabidopsis leaves (WT and *uvr8-6*). Qualitative analysis of flavonoids was performed using TLC. Plants were grown with and without UV-B (1 µmol $m^{-2}s^{-1}$) (indicated by -UV-B and UV-B respectively). Half the plants were cold acclimated in the light for 24 h at 4 °C with and without UV-B before tissue collection. Plants cold acclimated are indicated in the x axis with '4' and plants grown at 20 °C without cold acclimation are indicated by '20'. The flavonoids quercetin and kaempferol are indicated by orange and green bands. The higher the intensity of the colour bands indicates an increase in pigment levels. Letters A-G indicate TLC attempts adjusting the polarity of the mobile phase.

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